Cranfield University

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The impact of anecic earthworms on the dispersal of *Microdochium nivale* in amenity sports turf

School of Applied Science

Sports Surface Technology

MSc by Research THESIS

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Supervisors:

Prof. Karl Ritz
Dr Iain James
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This thesis is submitted in fulfilment of the requirement for the degree of Master of Science by Research.

Abstract

In sports surface management, integrated disease management (IDM) is useful in identifying the parameters for which disease can occur. The host, pathogen and environment are factors that are intrinsically linked for disease to manifest. When either of these causal components is not present then plant disease is prevented. Soil biotas have been implicated in the movement of pathogenic soil microbes through both consumption and ejection and by external contamination, the propagule attaching itself to the external wall of the earthworm. Soil biota could therefore be included as a causal factor in disease development. The aim of this thesis was to investigate the impact anecic earthworms have on the dissemination of *Microdochium nivale* in amenity sports turf.

An initial experiment to ascertain viability of *M. nivale* spores post ingestion through *Lumbricus terrestris* was performed. Sterilised soil pre-inoculated with *M. nivale* was fed to earthworms and propagules re-isolated from the faecal matter using a soil dilution technique. Results showed that 10% of viable propagules of *M. nivale* fed to earthworms survived the digestion process and were evident in the faecal matter (cast).

A turf microcosm experiment was established to record whether the casts containing propagules of *M. nivale* could lead to infections of Fusarium patch, the plant disease caused by *M. nivale* in amenity turf. Spiked casts (*M. nivale*) were placed in pots containing *Lolium perenne* and the incidence and severity of disease was recorded using image analysis. The conclusions were that the spiked cast material was no more infectious than spore solutions of *M. nivale* inoculated onto the plant material.

A final investigation of the effects the presence and absence of earthworms have on the dissemination of *M. nivale* propagules in turf grass was conducted. Turf microcosms containing *Agrostis stolonifera* were pre-inoculated with a spore solution of *M. nivale* in either the presence or absence of earthworms. Rate and progression of disease was recorded using image analysis, dispersal of propagules was assayed through leaf sampling in the microcosm. Results indicated that the presence of earthworms had a greater effect on both the manifestation of Fusarium patch, and the dispersal of *M. nivale* propagules than in the absence of earthworms.

This study has provided a contribution to understanding the interactions between *L. terrestris* and *M. nivale*. It is clear that earthworm interaction with *M. nivale* enhances the dispersal of viable propagules, potentially leading to fresh manifestation of disease. Recommendations regarding the management of *terrestris*, already considered a nuisance on fine turf due to its casting; would be to mitigate these earthworms in areas of intensively managed turf, whereby the advantages of high earthworm activity are neither necessary nor required.

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Saint Karl Ritz. I need say no more.....

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Chapter 1: Introduction

1.1 Background to the research

Sports turf managers are under increasing pressure to deliver high quality playing surfaces all year round. In the context of golf courses, perfectly manicured swards consisting of only the most suitable fine leaved grasses are considered a pre-requisite. The benefits of enticing new players and business are important in an industry that is becoming increasingly competitive. The intensity of turf management required to produce such surfaces relies on the extensive use of fertilisers, soil amendments and fungicides. All of which are necessary in delivering not only surfaces appropriate for play, but essentially, surfaces that are free from turf disease.

Over 3000 golf courses currently operate in the UK (Bartlett and James, 2011). With such enterprises spraying fungicides/pesticides on average 5 times per year, the UK market in these biocides is estimated at £6 million per annum (Irk, 2012, Syngenta; Personal Communication). The financial cost of fungicide use, although high for some facilities, is insignificant when compared to the environmental ramifications of such procedures. Fungicides pose a risk to the environment, moreso when on-site use leads to migration off-site through drift and surface run-off. Fungicides contaminating waterways also have adverse effects on the health of terrestrial and aquatic ecosystems. The accumulation of fungicides in the soil can also affect organisms present and reduce the fertility in the soil (Wightwick, *et al*., 2010). In 2003 Denmark initiated a ban on many fungicides including chlorothalonil and azoxystrobin, both heavily used in controlling fungal pathogens in amenity sports. In Germany, golf courses must apply for permission to spray such

materials. Prompt identification of turf disease is critical as the time required for application and consummation could be a period whereby the sward is irreversibly damaged (Moser, 2012; Personal communication). The repercussion of such legislation has led other countries within the EU to implement stricter controls regarding fungicide usage. A new EU directive (2009/128/EC) as of October 2009 was established which requires golf courses to develop action plans, including means for reducing pesticide use, and standards for water purity (Official Journal of the European Union, 2009).

A decrease in fungicide use is achievable upon comprehension of the parameters for which turf disease occurs. The pathogen must be present either in the thatch or the basal leaves (host), waiting for the correct environmental conditions in which to thrive. These factors fundamental for disease manifestation can be conceived as a 'disease triangle' (Figure 1.1), which illustrates how the host, the environment and the pathogen are intrinsically linked.

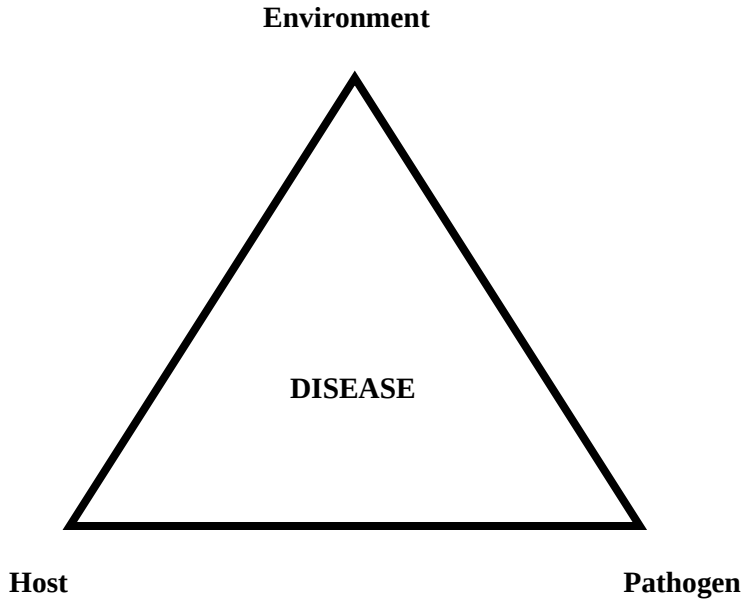

Figure 1.1 The disease triangle. Source: Arthur (1997).

The triangle identifies when disease may occur, requiring the interaction of a virulent pathogen, a susceptible host and an environment favourable for disease development. When either of these causal components is not present, then plant disease is prevented. All variable factors can be manipulated in the form of a well developed integrated disease management (IDM) programme, ensuring that outbreaks of disease are kept to a minimum. The environment can be managed to eliminate conditions conducive to disease manifestation through water management, aeration and fertiliser considerations. Turf managers can influence the turf composition in the longer term, although in most instances they have to manage what is manifest. *Poa annua*, a grass highly susceptible to plant disease is being replaced by fine leaved grasses such as *Agrostis stolonifera*. The inherent disease and stress resistance of bents and fescues play an important role in IDM, their low susceptibility to disease eliminating one of the causal components within the disease triangle. The disease triangle, although important in identifying the parameters in which disease can occur, fails to identify circumstances in which pathogen dispersal can be attributed to other determinants. Plant pathologists have elaborated on the disease triangle by adding more parameters relating to disease management, including human interaction in the form of plant husbandry through breeding and genetic engineering (e.g. Francl, 2007). Soil fauna, more specifically earthworms, have been implicated in the movement of viable soil microbes and plant pathogens through consumption and dispersal in their faecal matter, and by external contamination, the movement of propagules attached to the external wall of the earthworm (Parle, 1962; Agrios, 1980; Toyota and Kimura, 1994). Soil biota as vectors for plant pathogens could therefore be incorporated into the disease triangle as a

causal factor in disease development, leading to the conceptualisation of a 'disease tetrahedron' (Figure 1.2).

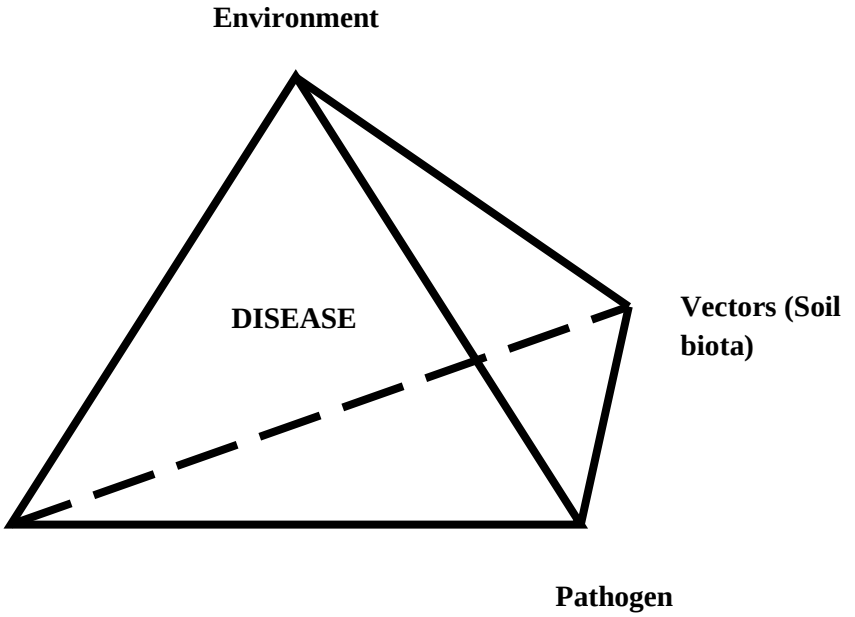

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Figure 1.2 The disease tetrahedron outlining a fourth causal factor of disease Source: Francl (2007).

The author's experience over 15 years in the turf industry has identified extensive earthworm activity coinciding with outbreaks of turf disease including *Sclerotinia homeocarpa* (Dollar spot) and *Microdochium nivale* (Fusarium patch). This could be coincidence, as field studies conducted by Lee (1985) have shown earthworms are at their most active when temperatures are between 10 and 15^{o} C; a temperature observed to be optimal for the establishment of Fusarium patch in amenity turf (Hsiang, 2007). There could however be a causal link between earthworm activity and increased disease occurrence as studies have identified earthworms as vectors for disease, in isolating fungal

plant pathogens from the gut and faecal matter of various ecological groups of worms (Toyota and Kimura, 1994; Moody, *et al*., 1995; Chhotaray, *et al*., 2011).

Earthworms play an important role upon the soil matrix. The burrowing of anecic and endogeic earthworms improves soil structure, creating open channels allowing gaseous exchange and aeration of the soil (Edwards, 2004). In agriculture, the encouragement of earthworms is important. As a result of the digestion process the earthworm's faecal matter is also high in nutrients, often resulting in increased crop yields where high earthworm populations are present. However, in a golf course environment this is an issue which is yet to be clarified. High populations of earthworms can create problems on amenity turf grass. The earthworms faecal matter (casts), when left on the surface can inhibit true ball roll, damage mowing equipment and restrict porosity on the soil surface (Potter, 2009).

A key question for the industry would be whether the encouragement or tolerance of earthworms in a golf course environment would be the same in light of a possible causal relationship between earthworm activity, pathogen dissemination and disease development. A blanket ban on fungicide applications in the UK is not realistic in the foreseeable future (Irk, 2012, Syngenta; Personal Communication), high fungicide costs and the environmental hazards attributed to such applications could however mean a reduction in fungicide use. Therefore, the future management of earthworms may prove important in sustaining sports surfaces to suitable standards. Limited information is available regarding faunal grazing in the soil matrix and the dispersal of plant pathogens. This thesis therefore investigated the interactions between soil biota and pathogen dispersal.

Chapter 2: Literature Review

2.1 Introduction

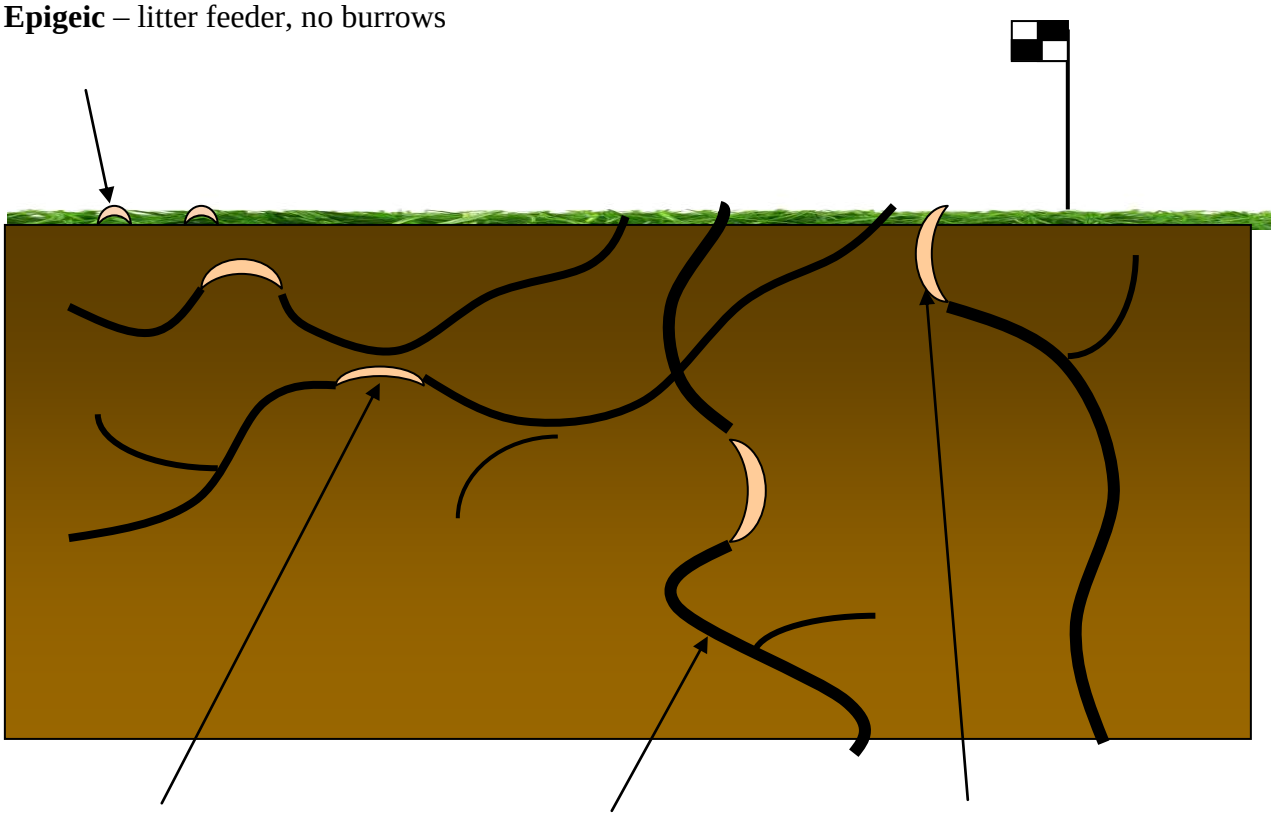
This review sets out to explore both the positive and negative impacts that earthworms have within the context of turf management. Emphasis will be placed on the earthworm's ability to act as a vector of both beneficial microorganisms and organisms detrimental to plant growth. The aim is to evaluate existing research to understand the various concepts, theories and methodologies and examine if, and where, there are gaps in knowledge pertaining to the research aim of this thesis. Special attention will therefore be directed towards earthworms and their dissemination of saprophytic plant pathogens which can have direct consequences with regards to turf management.

2.2 Earthworm taxonomy and morphology

Earthworms fall into the category of soil fauna and belong to the order Oligochaeta. Enumeration of earthworm species indicate as many as 8000 individual species worldwide placed into about 800 genera (Edwards, 2004). There are currently 25 species native to the United Kingdom and these can be classified within three distinct ecological groups according to their feeding habits, *viz*. anecic, epigeic and endogeic types (Figure 2.1). Anecic earthworms are species that feed on surface litter but live in vertical burrows within the soil matrix. They are larger in size than worms from the other groups and are considered problematic in sports turf management due to the turret casts they produce and leave on the surface (Bartlett, 2006). Anecic earthworms are capable of consuming large quantities of soil and leaf matter, as much as 30 times its own body weight per day. Epigeic earthworms live within the litter layers and as a result are prone to high mortality

rates due to predation, extreme temperatures and drought. Endogeic earthworms live and feed within the soil matrix. Although nominally a safer environment the nutritional value of food in such zones is typically lower than that found on the surface, therefore growth rate is not nearly as high as in anecic species (Lavelle, 2001).

Epigeic – litter feeder, no burrows



Endogeic – top soil dweller horizontal burrows

Burrows

Anecic - litter + soil feeder extensive vertical burrow

Figure 2.1 Functional traits and burrowing behaviours of the three major earthworm ecological groups

The differing ecological groups thus characterise earthworms and their distinct behaviour within the soil matrix. Although a range of species of earthworms will be covered in this literature review, focus will be primarily placed on the anecic types (casting earthworm) e.g. *Lumbricus terrestris* (Figure 2.2 & Table 2.1) due to the many implications this worm has for sports turf management.

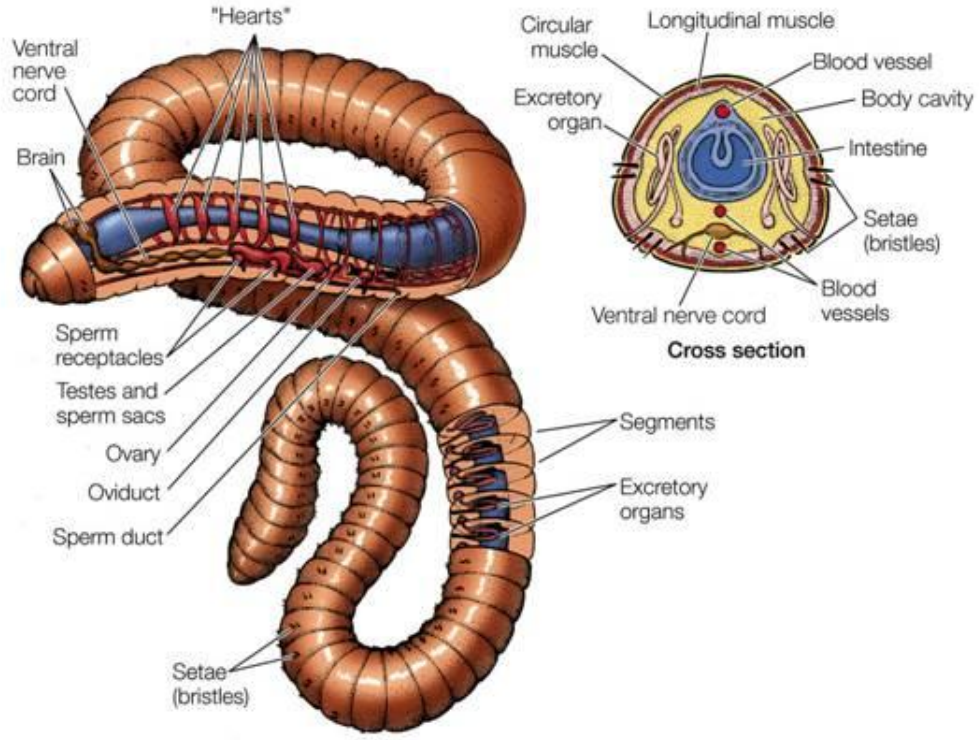


Figure 2.2 Morphology and anatomy of *Lumbricus terrestris* Source: *Life: The Science of Biology, 2007.*

Table 2.1 Scientific classification of *Lumbricus terrestris* L.

Scientific classification	
Kingdom:	Animalia
Phylum:	Annelida
Class:	Clitella
Subclass:	Oligochaeta
Order:	Haplotaxida
Family:	*Lumbricidae*
Genus:	Lumbricus
Species:	*L. terrestris*
Binomial name: *Lumbricus terrestris* Linnaeus, 1758	

2.3 The role of earthworms in soil structure dynamics

The ability of earthworms to break down organic matter, provide channels in the soil profile aiding both water infiltration and gas exchange, and to stimulate microbial activity increasing plant nutrient availability, are all well documented (e.g. Edwards, 2004). Organic matter is decomposed by the microflora that resides in the soil matrix. Soft plant and animal tissues are more easily utilised by both micro-organisms and the smaller fauna. Larger organic matter fragments such as leaves, roots and stems left on the soil surface are initially processed by the larger earthworms through ingestion and the enzymatic fluids located in their intestines (Edwards and Lofty, 1977). In a study conducted by Edwards and Heath (1963 cited in Edwards and Lofty, 1977); earthworms were found to consume

more oak and beech litter than all the other soil invertebrates together. Edwards (2004) reiterated that the anecic group of earthworms typically has capabilities to consume the total annual litter fall in woodland ecosystems. The consumption of matter by earthworms leads to the dispersal of the faecal matter, commonly known as casts. The cast material is nutrient rich particularly with respect to nitrogen, phosphorous and potassium in mineral forms hence easily available for plant uptake (Edwards, 2004).

The burrowing instinct of the endogeic and anecic earthworms greatly affects the structure of soil. The permanent burrows made by anecic earthworms aid water infiltration, drainage and porosity (Bartlett, 2006). Research cited in Edwards and Lofty (1977) reports that water leached the soil surface up to ten times faster in the presence of earthworms (Stockdill, 1966 cited in Edwards and Lofty, 1997). The burrows, often permanent due to the binding effects of the mucus present on the exterior wall of the earthworm, provide channels for plant roots, the faecal matter lined amongst these channels are a significant proportion of the nutrients absorbed by the plants.

Elmer (2009) commented that the population of earthworms present is a direct indicator of soil health. Population estimates in moist grassland and pastureland are upwards of 500 individuals m^{-3} although in the southern hemisphere populations can be as much as 2000 m^{-3} (Lavelle, 2001). With such high densities the earthworm's propensity to consume up to 30 times its own body weight per day in soil and organic matter, coupled with the movement this process involves, makes major contributions to soil development (Bartlett, 2006). As early as 1955, New Zealand introduced and actively encouraged certain earthworm species within agriculture in an attempt to increase field capacity, resulting in an

increase of 17% yield in some cases (Stockdill and Cossens, 1966 cited in Edwards and Lofty, 1977).

Agricultural practice leans towards actively encouraging earthworm activity. The naturalist and curate Gilbert White wrote 'Earthworms, though in appearance a small and despicable link in the chain of Nature, yet, if lost, would make a lamentable chasm….worms seem to be the great promoters of vegetation, which would proceed but lamely without them,…' (White, 1789)

Charles Darwin found the common earthworm fascinating. His last published work, *The Formation of Vegetable Mould through the Action of Earthworms*, published in 1881, was a revolutionary work based on earthworm behaviour and ecology. Darwin frequently described the earthworm as "beneficent gardeners" and "industrious ploughmen" (Darwin, 1881).

The beneficial impact that earthworms have on the surrounding environment is vast. The faecal matter expelled by the earthworm can be directly used in production systems. Vermicompost, a product utilising various species of worms to create a heterogeneous mixture of decomposing vegetable or food waste has a high nutrient content in forms easily utilised by plants. Vermicomposts can have indirect effects, for example, increasing symbiotic mycorrhizal association with roots and are also effective plant growth stimulators due to high levels of macro nutrients contained in the compost (Edwards, 2004).

2.4 The effect of earthworms on golf courses

It can be said that within the sports surface industry not all people agree that earthworms are indeed universally beneficial and should be encouraged. Jim Arthur, perhaps the most renowned green keeper of modern times described the casting earthworm (anecic) as "the worst of all pests", while proactively advocating the return of worm killers such as lead arsenate and chlordane (Arthur, 1997). His view and the views of others consider the casting earthworm a nuisance, the product of which, the cast, detrimental to maintaining adequate playing surfaces to an acceptable standard (Baker and Binns, 1988; Beard, 1973). Schread (1952 cited in Edwards and Bohlen, 1996) estimated that as many as 47 t ha^{-1} yr^{-1} of earthworm casts could be found on an average golf green, an indication of the scale of the problem associated with casting earthworms. The casts of anecic earthworms (Figure 2.3) are responsible for problems associated with fine turf. When left on the surface, the cast can be unsightly. Casts also provide seed beds for the establishment of weeds and grasses due to increased nutrients available in the cast compared to the surrounding areas (Edwards & Baker, 1992; Jefferson, 1958). Smeared casts are problematic in reducing water infiltration especially in the spring and autumn when earthworms are more active and evaporation is minimal (STRI, 1996). There is also the issue of general maintenance and mowing regimes. Casts contain mucus, a by-product of earthworm ingestion. The mucus has a tendency to become very hard when dry coupled with the inclusion of small aggregates and grit can result in precision mowers, especially greens mowers, requiring extended maintenance.

Figure 2.3 Typical cast material (turret) from *Lumbricus terrestris* in turf

In 2001 the Sports Turf Research Institute (STRI) compiled a survey completed by 190 golf courses within the UK. The results of the survey concluded that 81% of all golf greens were affected by *Lumbricus terrestris* and/or *Apporrectdea spp*. and that earthworms were recorded as the most widespread pest attributed to golf courses and their maintenance (Table 2.2; Mann & Newell, 2005). As of 2009 the STRI has received more enquiries regarding earthworm control than any other issues regarding turf maintenance (STRI; Personal Communication, 2012).

Table 2.2 Pest incidences reported as most common on a survey of golf greens in England, Ireland, Scotland and Wales in 2001.

Distribution by turf grass pest (%)		
Country	Total Replies	Earthworms
England	115	78
Ireland	20	85
Scotland	37	86
Wales	18	78

Source; Mann & Newell, 2005

2.5 The control of earthworms on amenity sports surfaces

Many methods have been developed over the years to ensure sports pitches remain free from earthworm casts. The need to eradicate the unsightly casts has left turf specialists in a quandary as to whether earthworms should be encouraged or neglected. On many sports pitches such as those for football or cricket, complete eradication is neither necessary nor desirable, the benefits of aeration from earthworm activity outweighing the effects casts have on playing conditions (Escritt and Arthur, 1948). Golf courses however, especially on golf greens, should preferably be free of casts. The effects on ball roll from cast material can have a detrimental effect on playability, in turn detracting from the enjoyment a golfer would normally get from playing the game (Beard, 1973).

Management of earthworms began in the late 19th Century when Darwin (1881) postulated that gardeners could use lime water to control earthworms in lawns. This initial

observation ensured turf specialists continued to identify possible chemical solutions that would control not only earthworm populations but surface casting. Chemical solutions fall into two categories. Expellants irritate the worm causing them to come to the surface such that they can. Examples of expellants include derris dust, potassium permanganate, formaldehyde, mustard and mowrah meal (Kirby & Baker, 1995). Mowrah meal was the most widely used expellant during the early 20th Century (Dawson, 1959). Derived from the seed of the butter tree of India, *Bassia latifolia*, mowrah meal applied at rates of approximately 250 g m^{-2}, apparently offered control up to two years (Kirby & Baker, 1995). The disadvantages of expellants during this time were evident, the collection of worms was labour intensive and areas containing a high level earthworm population could take days to clear. The amount of water required to 'water in' the expellant was logistically difficult. Golf courses using the expellant methods before modern irrigation systems were available suffered from frequent scorching of the grass, a significant side effect from heavy applications (Dawson, 1959). As a result chemicals that actively killed earthworms were preferred and used more frequently in controlling surface casting. The two most used chemicals were lead arsenate and chlordane. Chlordane was first registered for use as a worm killer in 1961 and trials were conducted by Lidgate (1966) in evaluating its performance, who concluded that chlordane was effective in controlling earthworms for a period of 2-3 years. Chlordane became the chemical of choice with greenkeepers although subsequent soil trials initiated the ban of chlordane in 1992 (in the UK) due to high levels of toxicity still found within the soil matrix and the risk to human health (BIGGA, 2011). In the USA researchers have indicated that high levels of chlordane still persist in certain areas due to the high levels of generic use in the 1970's and 1980's in treating termites.

Chlordane has been responsible for health problems including child cancers, leukaemia, infertility and neurological disorders (Sinclair, 1987). Lead or calcium arsenate was also banned in the UK in 1988, again on health grounds due to arsenates possibly being carcinogenic. Trials by Escritt (1955) concluded that calcium arsenate was very effective in providing control of earthworms but would not be suitable for areas of fine turf because of high levels of scorching. Arthur (1997) was of the opinion that pesticides such as lead arsenate and chlordane should never have been banned and the resulting fungicides labelled for earthworm control such as carbendazim non-effective. The use of worm killers effective for up to 7 years can carry certain environmental consequences. As a result of chemical applications populations of worms may never return to original numbers. Many pesticides/fungicides are toxic to animals, birds and aquatic life. Golf courses are highly managed habitats, the encouragement of indigenous species and natural fauna and flora are important in sustaining continued golf course development. The use of chemicals in controlling pests and diseases can adversely risk these ecosystems.

An alternative to chemical control is cultural control. Methods based on cultural control aim to manipulate the local environment of the turf and associated soil such that conditions are less conducive to the activity of earthworms. Methods of cultural control include the removal of the earthworms food supply. Studies have shown that boxing off grass clippings, a source of food for anecic earthworms, during mowing procedures significantly reduces earthworm casting (Edwards & Bohlen, 1996; Baker and Binns, 1998). Applications of acidic fertiliser have shown to decrease earthworm activity, possibly as a result of decreasing pH levels in soil (Edwards and Bohlen, 1996). Recent innovations

include the application of finely crushed glass on the surface areas of fine turf. While the glass is small enough not to damage mowing machinery, the abrasiveness of the glass particles is disliked by the earthworm and apparently restricts movement to the surface and the number of casts deposited (Nielson, Personal Communication, 2011).

The control of earthworms varies dependant on sports surface. In the context of a golf course, whether greens or fairways, earthworms are not desirable. Therefore, chemical or cultural control is an important aspect of golf course management.

2.6 Earthworms as vectors of soil microbes

Earthworms are not necessarily restrained to just physically improving soil structure or breaking down organic matter as reviewed previously. An underlying factor in the movement of earthworms is their ability to consume and carry soil microbes. Soil microbes are a large constituent of the soil matrix and are instrumental in many processes including breaking down organic matter into detritus (decomposers), developing beneficial symbiotic relationships with other organisms and fixing nitrogen. Soil microbes are an integral component of the earthworm's diet and consequently microbes ingested by earthworms can be disseminated via transit and faecal ejection.

Mycorrhizas, mutualistic fungi associated with the majority of plant roots (Smith and Read, 2007) play an important role in plant nutrition and allow the plant to uptake nutrients that would normally be unavailable whilst increasing plant tolerance to drought (Nelson, 1987 cited in Reddell and Spain, 1991). Arbuscular mycorrhizae (AM) are fungi which enhance

plant growth via affording enhanced nutrition and water acquisition. Reddell and Spain (1991) showed that earthworms can be vectors of viable propagules of AM fungi. They concluded that AM spores were capable of surviving passage through the earthworm gut in numbers requisite for further plant infection. Glasshouse experiments showed that AM spores and root fragments recovered from the casts of *Pontoscolex corethrurus* and *Diplotrema heteropora* maintained viability and initiated mycorrhizal infection on *Sorghum bicolor*. This was further elaborated on by Doube, *et al.* (1994), who affirmed the activity of earthworms in promoting the dissemination of varied amounts of beneficial soil microorganisms in the form of mycorrhizal fungi, rhizobia and pseudomonads.

Pattinson, *et al*. (1997) conducted a review of earthworms as vectors of beneficial mycorrhiza and in contrast to Reddell (1991) and Doube, *et al*. (1994), came to the conclusion that although earthworms have the capacity to disseminate, they found little evidence to support the idea. Pattinson, *et al.* (1997) were of the view that physical disturbances of the soil due to earthworm movement disrupted the hyphal network and reduced the infectivity of the fungus. They also concluded that fungal propagules of mycorrhizae did not survive passage through the earthworm gut, preventing the propagules from infecting plant material. Gange (1993) experimented further with CFU enumeration and found that previous research had underestimated the numbers of beneficial spores and fungal material found in the casts of *terrestris*. Gange's study placed emphasis on the enumeration of all propagules, not just spores, using the series dilution technique and most probable number method. Gange felt that the wet sieving method employed by researchers prior to his investigation was too coarse to retain all spores; non-spore propagules such as

hyphae could also be missed. Further evaluation confirmed that earthworm casts contain significantly higher amounts of fungal propagules in contrast to the surrounding soil.

Evidence suggests that earthworms are not solely vectors for mutualistic fungi but also saprotrophic fungi and plant root pathogens. Studies suggest that earthworms could in fact disseminate pathogens and spores that are detrimental to plant growth, which could in turn present serious issues in agricultural and horticultural practice as a result of significant decreases in productivity.

Khambata and Bhatt (1953 cited in Parle, 1962) had already made the assumption that pathogenic fungi such as *Pythium* and types of *Fusarium* could also be dispersed through the soil by earthworm activity.

Hutchinson and Kamel (1956 cited in Parle, 1962) investigated the viability of thick and thin walled fungal spores passed through the earthworm gut. Results showed that *Lumbricus terrestris* was capable of consuming several different species of fungi and through their burrowing traits increasing the dispersal within the soil matrix. Observations were further made that the dissipation of fungi was greater in the presence of earthworms than if they were absent.

Toyota and Kimura (1994) investigated the effects of earthworms in disseminating *Fusarium oxysporum*, a major disease affecting radish and banana crops. Cultures of *F. oxysporum* were fed to earthworms (*Phleretima spp*.) through inoculated radish disks. The casts were analyzed using a soil dilution plate method to enumerate viable propagules that

had survived ingestion through the earthworm gut. Results showed that the pathogen was present in 26 out of 28 casts, suggesting that further earthworm activity could in fact disseminate the propagules to a fresh environment and thus possibly culminating in a risk of new infection.

In contrast a study conducted by Elmer (2009) also pertaining to *F. oxysporum* experimented to identify the influence of earthworm activity on soil microbes and soil borne diseases of vegetables. Earthworms were introduced to soil pre-inoculated with *F. oxysporum* containing susceptible cultivars of asparagus and tomato. Results showed that an increase in earthworm activity alleviated the effects of the disease pathogen whilst dry plant weights were increased by 60-80% (Elmer, 2009). While both Toyota and Kimura (1994) and Elmer (2009) agree that earthworms do indeed reduce the amount of propagules in the soil environment, Elmer appears to advocate an increase in earthworm populations to negate the infectivity of pathogens already present.

Much research has been conducted to identify why earthworms apparently have the capacity to suppress disease. Edwards and Arancon (2004) took the view that the earthworms faecal matter is disease-suppressive due to high concentrations of nutrients which in turn support a varied microbial community. Clapperton, *et al.* (2001) also identified that earthworm presence increased the total mass of phospholipids fatty acids in the soil, indicating a large and active microbial population. Large microbial populations compete with pathogens for carbon and nitrogen, and in some cases microbes are antagonistic to specific pathogens, in turn contributing to disease suppression. Friberg, *et al*. (2005) stated that the groups of organisms collectively known as soil fauna were of the

utmost importance in regulating the survival and dissipation of plant pathogens and further still, augmenting soil conditions for the establishment of favourable soil fauna to control plant pathogens would be considered feasible in sustaining optimum conditions within agriculture. The idea that earthworm activity could affect the pathogenicity of certain pathogens was further developed by Wolfarth, *et al.* (2011). They conducted an experiment to study the effects certain species of earthworms had on the reduction of *Fusarium* biomass, measured through quantification of *Fusarium* protein equivalents (FPE) and deoxynivalenol (DON) content in wheat under field conditions. DON contamination of grain can lead to substantial crop losses in cereal based feed and can be toxic to both animals and humans. Results indicated that the earthworm spp. *Lumbricus terrestris* was primarily more attracted to the DON-infected wheat (straw) as evident in the amount of contaminated straw consumed as opposed to the control straw on offer. Recognizing that anecic earthworms were responsible and played an important role in the control of toxinogenic fungi.

A reduction of fungal pathogens as a consequence of worm ingestion is not always supported. Studies have shown that pathogen numbers increase while passing through the worm gut. Satchell (1983) commented that although some organisms declined in numbers after passage through the intestine, there was evidence to suggest that other organisms proliferated, and as a result passed through the gut unharmed. Parle (1962) embarked on a comprehensive study of earthworm nutrition at Rothamsted (Research Experimental Station), and contrary to many researchers concluded that actinomycetes and bacteria, but not fungi increased in numbers during passage through the earthworm gut. Parle concluded

that the mechanical action of the worms intestines broke down any organic matter ensuring the material was more susceptible to microbial attack.

Chhotaray, *et al.* (2011) looked into the diversity of bacteria and fungi contained within the cast of the epigeic tropical earthworm *Glyphodrillus tuberosus.* Isolation of various fungi and bacteria was achieved using the serial dilution method. Plates were then incubated at the temperatures required for optimum recovery. Results showed that fungi isolated from the casts included those from the *Fusarium* species. Although possibly present, *M. nivale* was not evident, due to incubation temperatures of 28^{o}C considered or likely too warm for its retrieval. Results also indicated that microbial numbers were significantly higher in the gut section of the worm and in the casts analysed compared to the un-ingested soil. Chhotaray, *et al.* (2011) concluded that fungi passing through the intestine were subjected to a beneficial environment capable of providing nutrients assisting growth and activity of various microbes. This is reiterated previously in the review by various authors identifying earthworms as a mechanism for both propagule dispersal and enhancement.

The ability of the earthworm to disseminate fungal pathogens requires the propagule to survive ingestion through the earthworm gut. The digestive enzymes and intestinal fluids within the earthworm gut have the capacity to affect the viability of some propagules (Moody, *et al*., 1995; Edwards and Fletcher, 1988). The viability of spores post-ingestion relies on many factors including spore size, pigmentation, cell structure and shape (Edwards and Fletcher, 1988). Therefore determining which species can survive would give a greater understanding of the connection between earthworm activity and turf disease outbreaks.

2.7 Feeding habits of earthworms

Investigation into the feeding habits of earthworms observed certain relationships between their role of vectors of pathogens and their dietary requirements. Bonkowski, *et al.* (2000) looked at the food preferences of earthworms for soil fungi and concluded through various experiments (offering differing fungi to earthworm spp.) that there was a certain trend towards fungi that offered more nutritional value. Among the fungi offered were *Fusarium nivale*. *Fusarium nivale* was identified as being one of the more preferred food sources available to the earthworms especially *terrestris*. Bonkowski *et al*. further concluded that dark pigmented fungi and plant pathogens were favourable along with fast growing saprotrophs. Edwards and Fletcher (1988) however observed through experimental studies that fungi such as *Fusarium* spp. *Aspergillus* spp. and *Penicillium* spp. could produce toxins poisonous to earthworms, and after consumption of these fungi, effects were reflected in high earthworm mortality rates.

There is thus evidence that earthworms certainly feed selectively. The dietary requirements of earthworms will have effects on the pathogenicity and spread of fungal pathogens as a direct result of exposure to the intestines and dispersal through extended activity.

2.8 *Microdochium nivale*

Microdochium nivale (fr. Samuels and Hallett, 1983), the causal agent of *Microdochium* patch, *Fusarium* patch (*nivale*) or pink snow mold is a fungal plant pathogen (Hofgaard, *et al*., 2006) primarily affecting regions in the northern hemisphere (Iriki, *et al.,* 2007). The species is psychotropic i.e. has the ability to grow at temperatures of 7^{o}C or below. The disease was first identified in England on golf greens in 1931, and apart from the name very

little has changed in the last 80 years with regards to controlling what has been described as the most prevalent of all turf diseases (STRI, 1996). The taxonomy of the organism has been dynamic (Table 2.3). *M. nivale* was first described in 1825 as *Lanosa nivalis* due to similarities in spore characteristics to those of other Fusarium species. In the late 19th century the name was changed to *Fusarium nivale* providing the common disease name *Fusarium* patch. Subsequently mycologists noticed the pathogen lacked certain characteristics in conidia morphology shared with other species of *Fusarium*, while noting comparisons with another species of *Microdochium*. The name was finally changed in 1983 though *Fusarium nivale* (patch) is still ubiquitous in its use today.

Table 2.3 Synonyms of *Microdochium nivale*

Anamorphs
Lanosa nivalis Fries 1825
Fusarium nivale (Fries) Soraurer 1901
Fusarium nivale Cesati ex Berlese & Voglino 1886
Fusarium hibernans Lindau 1909
Gerlachia nivalis (Cersati ex Sacc) Gams & Müller 1980
Microdochium nivale (Fries) Samuels & Hallett 1983

(Tronsmo, *et al*., 2001)

Recent research pertaining to molecular analysis of *M. nivale* has recognized two distinct varieties of which have been elevated to species status. Prior to 2005 *M. nivale* was categorised as a single species. However Glynn, *et al.* (2005) provided phylogenetic evidence of two different species of '*nivale'* based on biological differences relating to morphological characteristics, predominantly the size of and number of septae within the conidia. *M.* nivale *var. nivale* (Figure 2.4) has 1-3 septae where as *M. majus* has 3 septae. The two species have distinct differences regarding pathogenicity in plants. Both species affect wheat and whilst '*majus'* is considered to induce more severe symptoms, it does not however affect turf grass (ISTA, 2012). Therefore this study will focus solely on *M. nivale var. nivale* as the pathogen primarily affecting the sports surface sector.

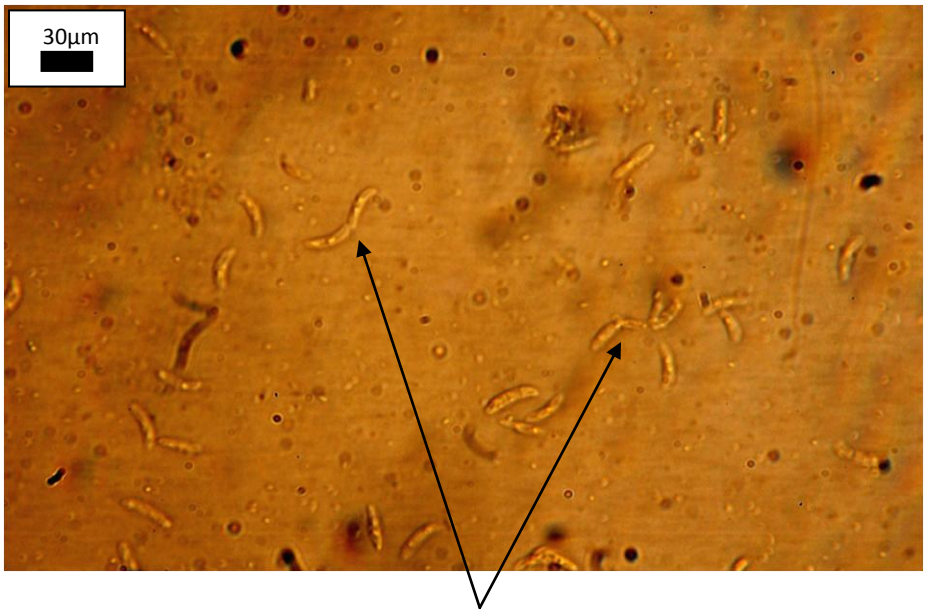


Figure 2.4 Macroconidia of *M. nivale var. nivale*

Identification of *M. nivale* on the golf course is relatively straight forward to the experienced greenkeeper mainly due to the prolificacy of the disease. While all turf grasses are susceptible, *Poa annua* tends to be most affected. Arthur (1997) states, that most golf courses will have an outbreak of *M. nivale* at some stage during the year. This was reiterated by the same survey conducted by Mann and Newell (2005) with regards to not only worm castings but also the prevalence of *Microdochium* patch on golf courses within the UK. Results concluded that 98% of greens conducted in the survey had encountered outbreaks of *Microdochium* patch within the last year, elevating its status to the primary disease concern to the turf specialist. Although not considered as problematic or devastating as some turf diseases, serious outbreaks on golf greens can have large repercussions and significantly affect playing surfaces. An example of this was at the 1995 British Masters Golf tournament at Collingtree Park Golf Club, Northamptonshire. The greens were so severely affected by *Microdochium* patch that greens staff dyed the putting surfaces green to conceal the lack of grass present (Anon. 2012).

M. nivale is persistent due to its ability to infect plants and maintain pathogenicity within a wide range of environmental conditions. Most notably, *M. nivale* sporulates best at temperatures of below 18^{o}C (Nelson, *et al.*, 1983). The influence of temperature on pathogenicity is considered a significant factor. Hudec & Muchova (2010) outlined this in a study conducted to identify the effects that differing temperatures have on the spread of *Fusarium sp*. They concluded that although the majority of *Fusarium* spp. grew fastest at 25^{o}C, *F. nivale* was the only disease that realised optimum growth at 15^{o}C. Arsvoll (1975) and Okuyama, *et al.* (1998) both concurred that maximal growth of *M.nivale* was

established at between 15 and 20^{o}C. Therefore, special attention can be given to *M. nivale* within the UK due to the conducive environmental conditions.

Figure 2.5 *Microdochium nivale* in early stages on *Poa annua* (annual meadow grass) Source: Hsiang, 2007

M.nivale starts as small orange/brown circular spots on the leaf (Figure 2.5), progressing quickly in a matter of days to areas seen in the sward with a diameter of approximately 50 mm. The areas increase rapidly under cool and/or humid conditions until in extreme cases the whole area can be affected (Figure 2.6). The UK winter and spring provides ideal weather conditions for the emergence *of M. nivale*, with low temperatures barely above freezing and highs of no more than 10 to 15^{o}C (Arthur, 1997).

Figure 2.6 *Microdochium nivale* (snow mold) in later stages on a golf green predominantly *Poa annua* Source: Kimberley Golf Club (2011)

In the context of golf courses, the dissemination of *M. nivale* to new environments e.g. golf fairways to golf greens, can typically be a result of transference of the pathogen on golfer's shoes, golf bag trolleys and golf course machinery.

At optimum *M. nivale* growing conditions and at the first signs of infection, accepted practice within the greenkeeping industry is to wash golf course maintenance machines carefully after each use; in extreme cases, where disease is detrimental to playability e.g. on

golf greens, standard practice is to wash machinery between greens cutting, although not usually adhered to and time consuming, this procedure has shown beneficial effects in the control and spread of the disease.

Microdochium attacks the plants via mycelia, conidia and ascospores. According to Parry (1995) the main threat of *Microdochium* is through wind-dispersed ascospores. The ability of climatic conditions to influence the threat of disease can give turf specialists very little warning of when an outbreak may become apparent. Differing views include those of Domsch, *et al.* (1980) who believes that the source of the infection is soil-borne through the spread of condia and hyphae; hyphae having strong saprophytic ability. Either way, the ability to forecast such outbreaks is dependent on many variable factors. Disease-forecasting systems have been developed for agriculture in identifying the potential loss of crops when the pathogens interaction with the host and the environment are such that disease develops. Although there are disease forecasting systems used in agriculture. in the turf industry, as yet, no system is applicable for *M. nivale* (VIPS, 2005).

2.9 Control of *Microdochium nivale*

Integrated disease management (IDM) is a model used within the agricultural and horticultural industry and is defined as 'The complementary use of cultural, biological and chemical methods to maintain disease at an acceptable level' (Arthur, 1997). IDM places importance foremost on cultural methods used in disease prevention. Cultural methods can include increased aeration, decreased irrigation, and the removal of thatch and dew, all beneficial in reducing surface moisture, an environmental factor favouring disease

development. The application of fertilisers and soil amendments has a major preventative effect on disease outbreak due to the hardening effects on the plant leaf (Arthur, 1997). Care must be taken that fertiliser applications with high amounts of nitrogen are not used late in the season. New leaf growths resulting from excess nitrogen are more susceptible to disease infestation and the sugars and amino acids exuding from newly developed leaves aid spore germination and growth of some pathogens. High nitrogen levels coming into the winter months is a prerequisite for disease outbreak and should be discouraged (STRI, 1996). Although cultural methods are desirable in controlling disease, these methods are not always effective. Biological control is considered the next step in disease prevention. Biological control is the use of one organism to out-compete another and is a method that relies of predation, parasitism and other natural mechanisms. At present there are few biological methods used in the turf industry. The problems of introducing biological controls are that the consequences often outweigh the initial benefits, as introduced controls can have an invasive effect (Arthur, 1997). Another biological method is the use of turf grass cultivars resistant to disease attack. Of all the grasses susceptible to attack by *M .nivale*, *Poa annua* is considered the most vulnerable. The species' inherently weak growth, especially when subjected to high levels of nitrogen encourages outbreaks of *M. nivale*. Further still, the shallow root system of *P. annua* requires increased levels of irrigation which in turn creates an environment suitable for disease development. Implementing the use of turf grass species and cultivars resistant to invasion are considered to be an expensive alternative to cultural methods and the re-seeding and renovation of golf greens not viable due to time constraints during construction, loss of revenue and initial cost.

When all cultural and biological methods have been exhausted, only then is the use of chemical control recommended (Hofgaard, *et al*., 2006). In countries where annual snow is prevalent, fungicide applications are made two to three weeks prior to the first snow fall. These applications normally consist of a tank mix containing both a systemic and contact fungicide. Fungicide applications tend to be administered when climatic conditions are conducive to disease development. Problems often associated with fungicide applications are the timing of the treatments, climatic conditions differ year on year and as a result many applications are in fact unnecessary (Hofgaard, *et al.,* 2006).

With the prolificacy of *M. nivale* on golf courses within the UK, the economic and environmental factors associated with fungicide treatments need to be considered. The cost of fungicide applications depend on the amount of area treated and the chosen chemical used. As a rule systemic fungicides tend to be more expensive and a single application for eighteen golf greens as of 2012 will cost approximately £800 (I'Anson, Personal Communication, 2012). When taken into consideration golf courses will on average spray 4-5 times per year and in some cases as much as 8-10 times per year, this can have significant effects on the fiscal demands placed on golf course enterprises. Also, the continued use of the same systemic fungicides can lead to some diseases becoming immune; resulting in a limited choice of fungicides in the future. To date there are currently severe restrictions in much of Europe pertaining to the use of fungicides. Many of the active ingredients found in fungicides are toxic to aquatic life, particularly problematic on golf courses with water features or irrigation lakes.

2.10 Conclusions

This review has identified earthworms within their own environment and the roles they play in not only improving soil structure but more specifically, their roles as vectors of beneficial and saprophytic fungi. Various views support the idea that earthworms have the ability to spread disease pathogens enabling wide spread infection. Contrary to this there is also the view that the earthworm could be a means to suppress further infection, and in fact alleviates many of the issues faced when dealing with crop and turf disease. Research has indicated that inoculated soil containing spores of differing fungi were dispersed moreso through the soil when earthworms were present than absent. Further still selective feeding of fungi is evident due to higher levels of specific fungal propagules in worm casts compared to the surrounding soil.

The question then is, should we encourage soil faunal activity due the positive effects such as improving soil structure, increasing field capacity and the ingestion of dead and decaying organic matter? Or, should we be encouraged to eliminate or decrease earthworm populations to alleviate the possibility of pathogen dissemination and the negative impacts that earthworm casting has in amenity sports? Furthermore, is there a risk of pathogens such as *M. nivale* being positively dispersed by soil fauna within a golf course environment to an extent that it could become possibly problematic with regards to maintaining a healthy sward?

2.10.1 Research aim, objectives and hypotheses

The primary aim of this study is to investigate links between the anecic earthworm *Lumbricus terrestris*, and the dissemination of the plant pathogen, *Microdochium nivale var. nivale*. The thesis will investigate the impact of *L. terrestris* as a causal agent in the establishment of *M.nivale* in amenity turf grass. The specific objectives and associated hypotheses are then:

2.10.2 Research objectives and associated hypotheses

1. To ascertain viability of *M. nivale* propagules in worm cast material post ingestion through *Lumbricus terrestris*. The hypothesis tested will be 'Propagules of the plant pathogen, *M. nivale*, present in earthworm casts will be viable'
2. To establish if viable *M. nivale* present in worm casts is pathogenic. The hypothesis tested will be 'Earthworm casts containing propagules of *M. nivale* when introduced into turf will lead to manifestations of Fusarium patch'
3. To establish if earthworms disseminate *M. nivale* in turfgrass and accelerate disease development. The hypothesis tested will be 'Propagule dispersal of *M. nivale* and disease manifestation from a point inoculum is greater in the presence of *L. terrestris'*
4. To synthesize results and consider implications in relation to the future management of earthworms in a golf course environment.

2.10.3 Summary of experimental approach and thesis outline

For a comprehensive investigation to determine the effects anecic earthworms have on pathogen dissemination, all hypotheses will be tested following a logical progression: i) To test viability of spores following passage through an earthworm ii) To establish whether spores present in the faecal matter instigate infection in amenity sports turf iii) To understand the impact anecic earthworms have on the advancement of disease manifestation and propagule dispersal in amenity turf.

To ascertain viability of the spore post ingestion is addressed in Chapter 3 where in vitro feeding of *M. nivale* to *L. terrestris* will enable enumeration of viable propagules through a series dilution technique. A turf microcosm experiment with amenity grass inoculated with spiked and control casts, plus differing loads of *M. nivale* inoculation to establish whether earthworm casts are indeed potential contagia, is investigated in Chapter 4. Chapter 5 identifies the role of earthworms as actual vectors of disease. A synthesis is then presented in Chapter 6, including recommendations as to the appropriate management of earthworms in light of findings. This chapter also highlights the contributions that this thesis has made to current knowledge, whilst identifying new questions for research and further work recommendations beneficial to future study.

Chapter 3: To ascertain viability of *Microdochium nivale* post ingestion by *Lumbricus terrestris.*

3.1 Introduction

In this first experiment the main aim was to establish whether spores of *M. nivale* survived passage through the earthworm gut and subsequently maintained viability. This would be a prerequisite if worms were to contribute to any increased risk of disease propagation in turf grass. Pathogens that survived earthworm ingestion could benefit from the resources found in a new environment contributing to increased disease development. The approach was to feed earthworms (*L. terrestris*) spores of *M. nivale in vitro* and ascertain their viability post ingestion.

Given precedent studies regarding the viability of spores post ingestion through anecic earthworms, it was postulated that spores do survive the digestion process. This was examined by testing the following hypothesis: **'Propagules of the plant pathogen, *Microdochium nivale,* located in earthworm casts will be viable.'**

3.2 Materials and methods

3.2.1 *Microdochium nivale*

A culture of *M. nivale var. nival*e was donated by Dr Ruth Mann from the Sports Turf Research Institute (STRI). The original isolate was sub-cultured onto potato dextrose agar (PDA) (Oxoid) and maintained at 18^{o}C in the dark for 14 days to produce sporodochia. A spore solution was prepared by flooding the plates with sterile distilled water (SDW) and gently scraping the surface with an L-shaped spreader, encouraging the dispersal of sporodochia and liberation of spores. The solution was then poured into a 30 ml vial and

topped up with SDW. The number of conidia was ascertained using a haemocytometer and microscopic examination.

3.2.2 Earthworms and soil

Adult earthworms of the species *L. terrestris* were donated by Bleak Hall sports shop (Northampton, UK) and identified using the OPAL Key to common British earthworms (Jones and Lowe, 2011). The earthworms were rinsed under tap water anterior end first and kept in 90 mm diameter Petri dishes containing wet filter paper to avoid desiccation. The earthworms were left for two days to allow for excretion of contents within the earthworm gut, rinsed again in SDW and left in Petri dishes for a further 24 hours to allow for final evacuation of the earthworm gut.

Top soil used for the experiment was obtained from Boughton Loam (Telford, Northampton) and classified as 3 mm screened and sterilised sandy loam with a particle analysis of 75-18-7 % (sand, silt and clay respectively). The soils pH was 7.2 with an organic matter content of 3.3% (loss-on-ignition method). The soil was macroscopically cleared of organic plant residues and sieved (2 mm) prior to autoclaving at 121^{o}C for 15 minutes. The soil was then stored in airtight containers at 4^{o}C until ready for use.

3.2.3 Experimental design and procedure

The experimental units comprised three oblong polypropylene microcosms measuring 250 x 180 x 90 mm including lids. Three treatments were established: (i) Microcosm containing bait soil (5 g) inoculated with a spore suspension of *M. nivale;* (ii) Microcosm containing

bait soil (5 g) inoculated with *M. nivale* plus an earthworm (*Lumbricus terrestris*); (iii) Microcosm containing a control soil (5 g), un-inoculated, plus an earthworm (Figure 3.1).

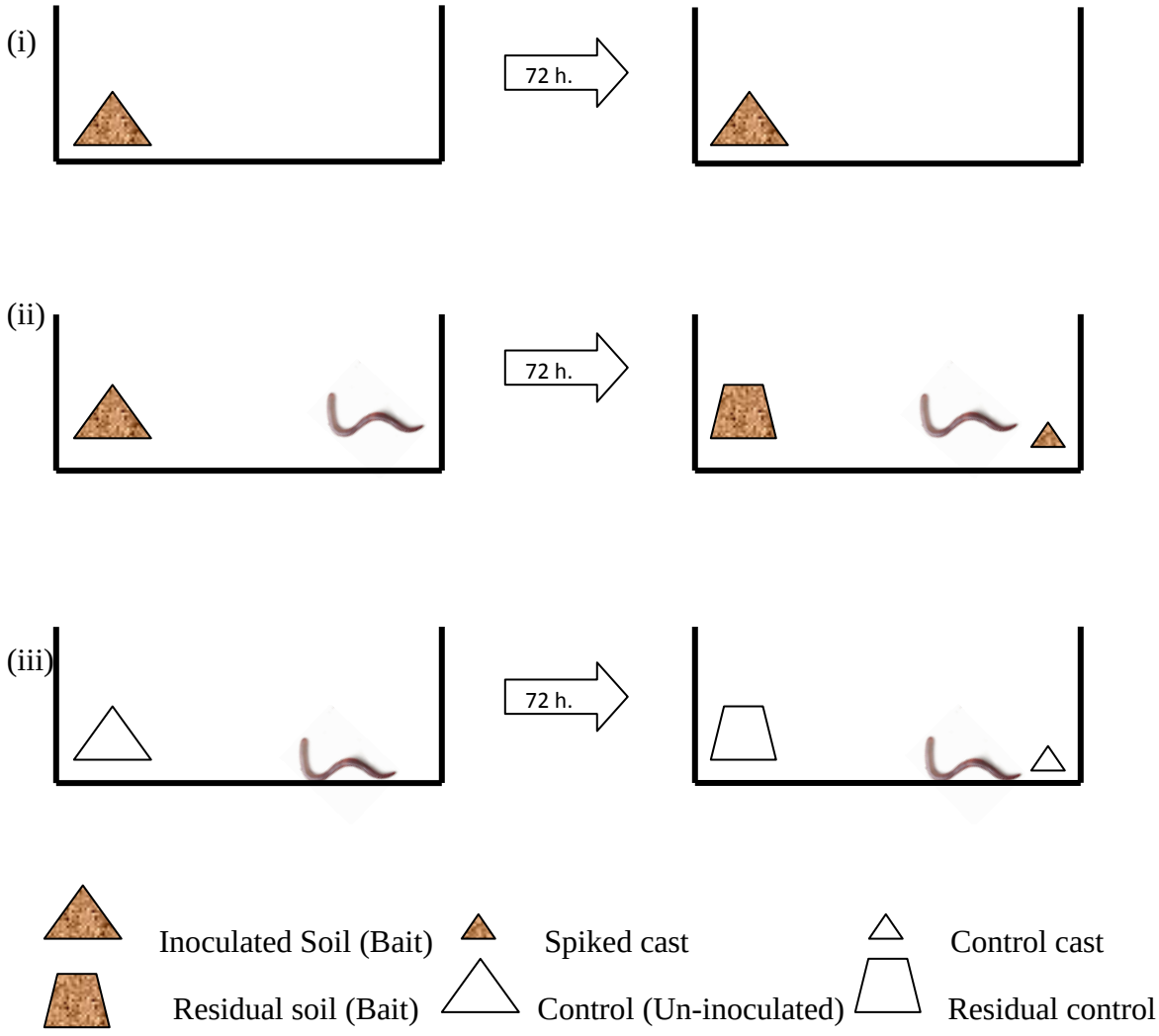


Figure 3.1 Experimental design to ascertain viability of *M. nivale* following passage through an earthworm over a 72 hour period

Sanitized earthworms were placed in stream sterilised microcosms containing 5 g steam sterilised soil initially inoculated with 3 ml SDW as a hydrating agent. Aliquots (1 ml) from the *M. nivale* spore solution with a concentration of 1.7 x 10^6 spores/ml were inoculated onto the soil. A weighing boat containing 75 ml SDW was added to the microcosm to ensure the earthworms did not dry out during the duration of the experiment (Figure 3.2). Perforated lids were firmly attached to the microcosms which were then placed into an incubator at 10^{o}C for a period of 72 hours. Five independent replicates of each treatment were established.

Figure 3.2 Overview of microcosm system containing earthworm (E) plus *M. nivale* spiked soil (S)

The microcosms were inspected daily to ensure that the earthworms were alive; consuming the material on offer and that humidity was adequate, with the addition of SDW when required.

After 72 hours all cast material was aseptically collected from all treatments. Samples (circa 1 g) of soil from the treatments containing no earthworm and approximately 1 g of the residual soil left by the earthworms were also sampled. Sample material taken from each microcosm was not homogenised, populations of *M.nivale* were estimated using the soil dilution method and CFU numbers were determined in individual samples. Diluted samples were spread onto PDA plates containing 50 mg/l chloramphenicol to inhibit bacterial growth. Triplicate plates were plated from each dilution and kept at 20^{o}C (dark) and inspected daily for the growth of fungal colonies. Identification of *M. nivale* was established visually using pictorial and morphological keys found in Nelson, *et al*. (1983) and Rayner (1970; Figure 3.3).

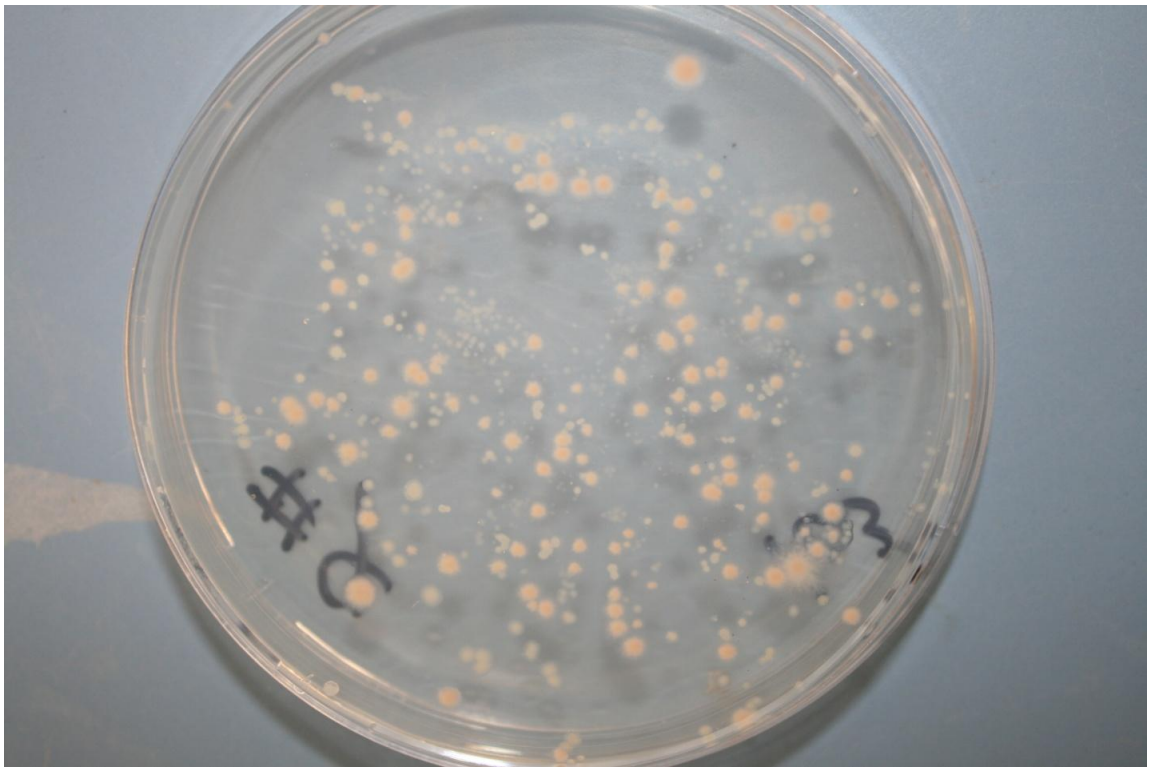


Figure 3.3 A PDA plate (diam. 90 mm) displaying *M. nivale* after soil dilution technique with dilution factor 10^3

To provide a comparison between the numbers of spores originally inoculated per gram soil and the numbers of CFUs isolated in the sample material, the remaining dilutions were converted to dry weight. This was determined by filtration. Dilutions were poured onto pre-dried and weighed 1.2 μm filter paper (Whatman), oven dried and re-weighed. Using the numbers of CFUs enumerated from the sample material and the dry weight of material from the soil dilutions, the CFU count was represented on a CFU g^{-1} dry matter basis.

3.2.4 Statistical analysis

All data were first checked for normality and homogenous variance (Anderson-Darling). One way analysis of variance was used to compare means. A post-hoc Tukey test was then applied. In text below, values are reported as means ± standard error.

3.3 Results

On opening the microcosms to collect cast and soil material, it was evident that the earthworms had been active in feeding on the initial material offered. Soil was distributed widely over the microcosm and evidently present as cast material. There was no significant difference in the mass of cast material collected from microcosms containing *M. nivale* and microcosms containing the control soil ($P>0.05$); overall mean weight was 42±18 mg.

Figure 3.4 shows the mean number of viable CFUs found in 1 g^{-1} dry weight of soil before and after passage through the earthworm gut. In the control microcosm containing earthworms and no *M. nivale*, analysis of the control cast showed no evidence of *M. nivale*. Some fungal species were evident, though in very low concentrations. Control earthworms produced casts with a range of 8-10 colonies per plate.

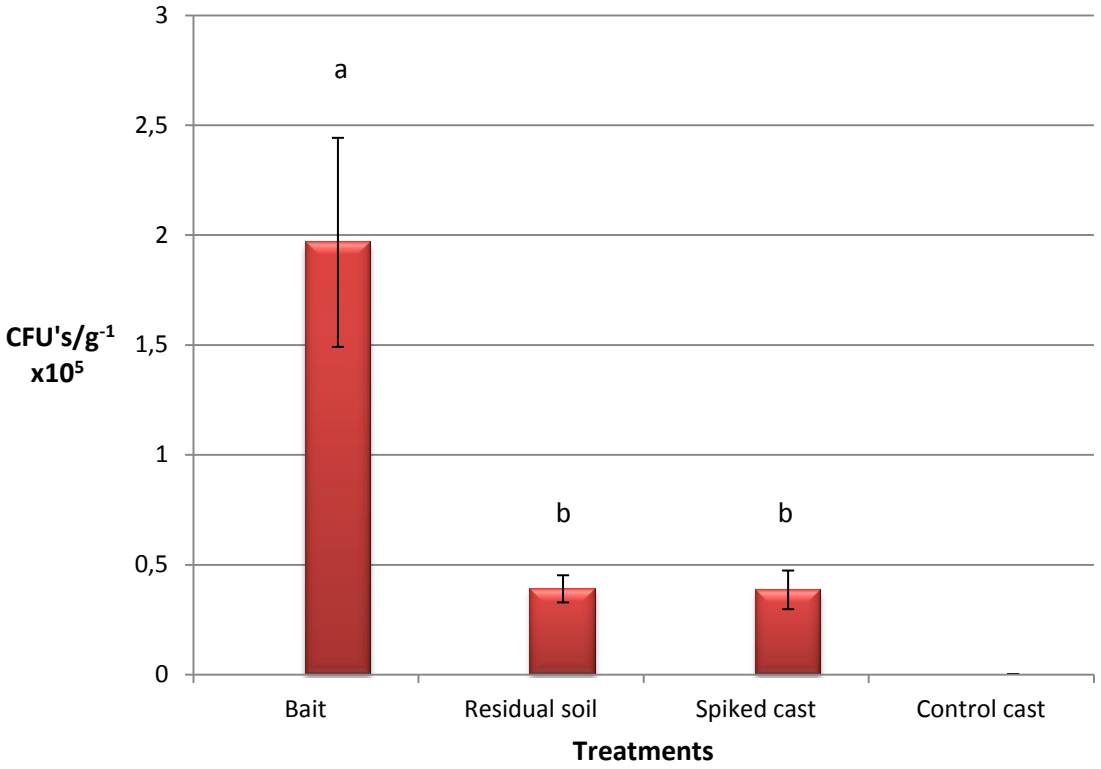


Figure 3.4 Concentrations of CFUs isolated in material before and after passage through a worm. Bars show arithmetic means (n=5) including SE bars. Letters denote homogenous groups at P=0.05, based on ANOVA of log-transformed data.

When comparing the number of CFUs isolated in the bait with the residual soil sampled and spiked cast there was a significant smaller concentration of CFUs isolated in the latter ($p<0.05$). There was no significant difference identified in the number of CFUs isolated from spiked casts compared to the residual soil from the same microcosms ($P > 0.05$).

3.4 Discussion

This study clearly demonstrates the presence of viable propagules of *M. nivale* in the earthworm's faecal matter following ingestion of some spores in a soil matrix. It is clear that spores survived the digestion process within the earthworm and that the enzymatic fluids and mechanical process in the gut had little effect on the viability and concentration of spore numbers. This is consistent with the findings of Toyota and Kimura (1994) that the earthworm is capable of disseminating viable propagules of plant pathogens.

The experiment design here relied on the control microcosm, containing soil un-inoculated with the presence of an earthworm, showing no evidence of *M. nivale* on analysis of the control casts. The results showed this; therefore it can be asserted that the sanitisation of the earthworms and sterilisation of the soil was sufficient to ensure no background levels of *M. nivale* could affect the interpretation of results where the inoculation of *M. nivale* was a primary factor.

The results also show that the addition of an earthworm lowered the concentration of fungal spores found when sampling both the residual soil not ingested by the earthworm and on analysis of the spiked cast material. There was a significant decrease in concentrations detected compared with the bait. On examination of the residual soil the interaction of the earthworm in sourcing food may have led to fungal material, in the context of this experiment, spores of *M. nivale*; being selectively consumed due to its high nutritional value (Edwards and Fletcher, 1988) and high moisture levels provided by fungal biomass (Satchell, 1983). This experiment provided no choice as to the food on offer. However,

Bonkowski *et al.* (2000) indicated in food selection tests, of the fungi offered to a variety of earthworms, plant pathogens including *M. nivale* and *Rhizoctonia solani* were the most preferred food sources irrespective of worm species. This would therefore indicate that the fungus offered in this experiment was of a type normally selected by anecic earthworms. The lower concentrations of CFUs isolated from the residual soil compared to the bait could also be as a result of external contamination, whereby the propagules attached to the body wall of the worms (Agrios, 1980). On opening the microcosms it was evident that the soil had been thoroughly agitated by earthworm activity. Spores could have been dispersed anywhere the earthworm moved and therefore not analysed in the sample material. Although limited information is available on the transit of propagules as a result of external contamination, Toyota and Kimura (1994) noted in a study relating to the dissemination of soil-borne pathogens that spores were evident in the lined burrows formed by earthworms. They also recognized however, that the general dissemination of soil-borne pathogens through earthworm activity was a result of ingestion and ejection.

The addition of an earthworm contributed to a lower concentration of CFUs isolated in the residual soil compared with the bait. The addition of an earthworm did not however, contribute to a lower amount of CFUs isolated in the spiked cast when compared with the residual soil. There was no significant difference in the concentrations of plant pathogens isolated in either the residual soil or spiked cast. This indicated that although the inoculum load was reduced in the presence of an earthworm through feeding and general interaction, the concentrations of inoculums did not differ, or did not in the case of this experiment.

After the duration of the experiment casts were collected from the microcosms for further evaluation. Gut contents evacuated at a later date were not sampled. This could be considered a design flaw in the experiment as numbers found in the cast material may not be a true representation of the amount of CFUs ingested. Although it can be assumed that the casts collected contained only material from the microcosm due to the earthworm sanitisation process prior to the experiment, it is postulated that earthworms would first consume matter beneficial to its dietary requirements (Edwards and Fletcher, 1988), indicating that the first casts collected would likely provide samples containing viable spores.

The findings indicate that the hypothesis '**Propagules of the plant pathogen, *Microdochium nivale*, present in worm casts will be viable'** can be accepted. Although the interactions of earthworms with inoculated soil led to a general decrease in concentration of CFUs, it is confirmed that spores do survive the digestion process and viable propagules were identified in the earthworms cast material. Whether this cast material is a contagion for further disease development will be investigated.

Chapter 4: Assessing the incidence and severity of Fusarium patch, in *Lolium perenne* L., via infected worm casts.

4.1 Introduction

In the previous experiment (Chapter 3) it was established that some spores of *M. nivale* survived passage through the digestive system of *L. terrestris* and maintained viability. In terms of epidemiological consequences, the next critical phase would be for such soil to be confirmed as representing viable inocula to instigate infection of turf. Therefore, the aim of this experiment was to establish whether such spores, having passed through the gut of an earthworm (*L. terrestris*), had the potential to further infect amenity turf grass. Aside from the other factors attributed to disease development, pathogenic spore dissemination through cast material could lead to the prescription of management techniques in addressing the issues of high earthworm population, more importantly, defining the management issues relating to high level earthworm casting on sports surfaces.

Due to the ostensibly beneficial environment created by the earthworm cast in aiding spore survival, it was postulated that any propagules viable in the cast material would be potent in terms of inducing infection in turf, and was examined by testing the following hypothesis:

'Earthworm casts containing propagules of *Microdochium nivale* when introduced into turf will lead to manifestations of Fusarium patch'

4.2 Materials and methods

4.2.1 *Microdochium nivale*

A spore solution of *M. nivale* grown on PDA was prepared using the same method described in Chapter 3, section 3.2.1. The spore solution was adjusted to represent a count established by a haemocytometer of 1.7 x 10^6 spores/ml. Subsequent spore solutions of differing concentrations were necessary for this experiment and the original stock solution was diluted to produce concentrations ranging from 1.7 x 10^1 through to 1.7 x 10^5 CFUs/ml.

4.2.2 Amenity grass and pots

Turf microcosms were made from 70 mm internal diameter black PVC drainage guttering cut into 100 mm long segments. A plastic saucer with drainage holes was attached underneath each pot and secured using silicone (Figure 4.1). Pots were then filled with steam-sterilized, sieved (4 mm) sandy loam soil up to 10 mm below the rim. *Lolium perenne* L. Cv Romance, (Barenbrug, Bury St. Edmunds, Suffolk, England) was seeded into the microcosms at a rate equivalent to 20-30 g/$m^2$and then placed in a greenhouse, misting frequently with STW until germination had been achieved. Pots were then watered (STW) on an as-and-when required basis ensuring soil was kept moist. After establishment the plants were cut to a sward height of 15 mm. To minimize stress levels on the newly established seedlings, no more than 30% of the plant leaf was removed at any one time. After six weeks the microcosms were placed in a cold frame outside with the addition of greaseproof paper wrapped round each treatment and secured with an elastic band to help reduce contamination between treatments. The microcosms were conditioned for a further 7 days prior to treatment inoculation.

Figure 4.1 Overview of microcosm system

4.2.3 Earthworms and cast material

Adult earthworms of the species *Lumbricus terrestris* L. were procured from Carpin'Capers fishing tackle shop (Northampton, UK). Identification was confirmed using the OPAL Key to Common British Earthworms (Jones and Lowe, 2011). The sanitization of earthworms and the collection of spiked and control cast material was collected using the same method outlined in Chapter 3. Two treatments were involved in the collection of casts. Individual earthworms were placed in plastic containers measuring 250 x 180 x 90 mm (including lids) containing soil (5 g) inoculated with *M. nivale* ($1.7x10^6$ spores/ml). A further treatment containing earthworms and soil (5 g) not inoculated with *M. nivale* was included.

Earthworms were left to work the soil and after 96 hours all cast material was independently collected from each microcosm and weighed. From each treatment 0.5 g (fresh weight) of spiked (*M. nivale*) and control (no *M. nivale*) cast material was placed in a sterile Petri dish for use in microcosm inoculation. The remainder of the samples was analysed using the soil dilution technique (see Chapter 3, section 2.3), with triplicate plates to enumerate CFUs present in the cast material.

4.2.4 Experimental design

Individual turf microcosms containing *Lolium perenne* were inoculated with *M. nivale* in differing concentrations and methods. Nine treatments were established with four replicates of each, total n= 36; (Table 4.1).

Table 4.1 Treatments, descriptions and amounts of inocula for experimental procedure

#	Treatment	Description	Amount
1	WC+*Mn*	Spiked cast	0.5 g
2	WC-*Mn*	Control cast	0.5 g
3	1.7 x 10^1 CFU/ml	Spore solution (*Mn*)	0.5 ml
4	1.7 x 10^2 CFU/ml	Spore solution (*Mn*)	0.5 ml
5	1.7 x 10^3 CFU/ml	Spore solution (*Mn*)	0.5 ml
6	1.7 x 10^4 CFU/ml	Spore solution (*Mn*)	0.5 ml
7	1.7 x 10^5 CFU/ml	Spore solution (*Mn*)	0.5 ml
8	1.7 x 10^6 CFU/ml	Spore s. sprayed (*Mn*)	0.5 ml
9	Control	Control	0
Total	9		

WC = Worm cast *Mn* = *M. nivale* Individual replicates were coded A - D

4.2.5 Experimental procedure

The spiked and control cast material (0.5 g, Treatments 1 and 2) were introduced into the centre of each microcosm. Spore suspensions of concentrations $1.7 \times 10^1 - 1.7 \times 10^5$ spores/ml (Treatments 3-7) were dispersed by syringe into the centre of each microcosm. A spore solution (0.5 ml) from the original stock solution with a count of 1.7×10^6 spores/ml was added to 20 ml SDW and inoculated onto the grass using a hand held sprayer, the solution covering the entirety of the sward (Treatment 8). A control microcosm free of any inoculum was included in the treatments (Treatment 9).

Once the microcosms had been inoculated they were placed into a cold frame in a randomized block design. The cold frame was seperated into 4 equal blocks housing each set of replicates using bambo cane and cotton mesh to minimise cross contamination through air, wind and water (Figure 4.2). Four temperature loggers (Talk 2, Tinytag, Gemini Data Loggers Ltd, UK were placed at various points inside and outside the cold frame, recording temperature every ten minutes. The Perspex lid of the cold frame was closed during the duration of the experiment, except during watering, cutting and disease monitoring.

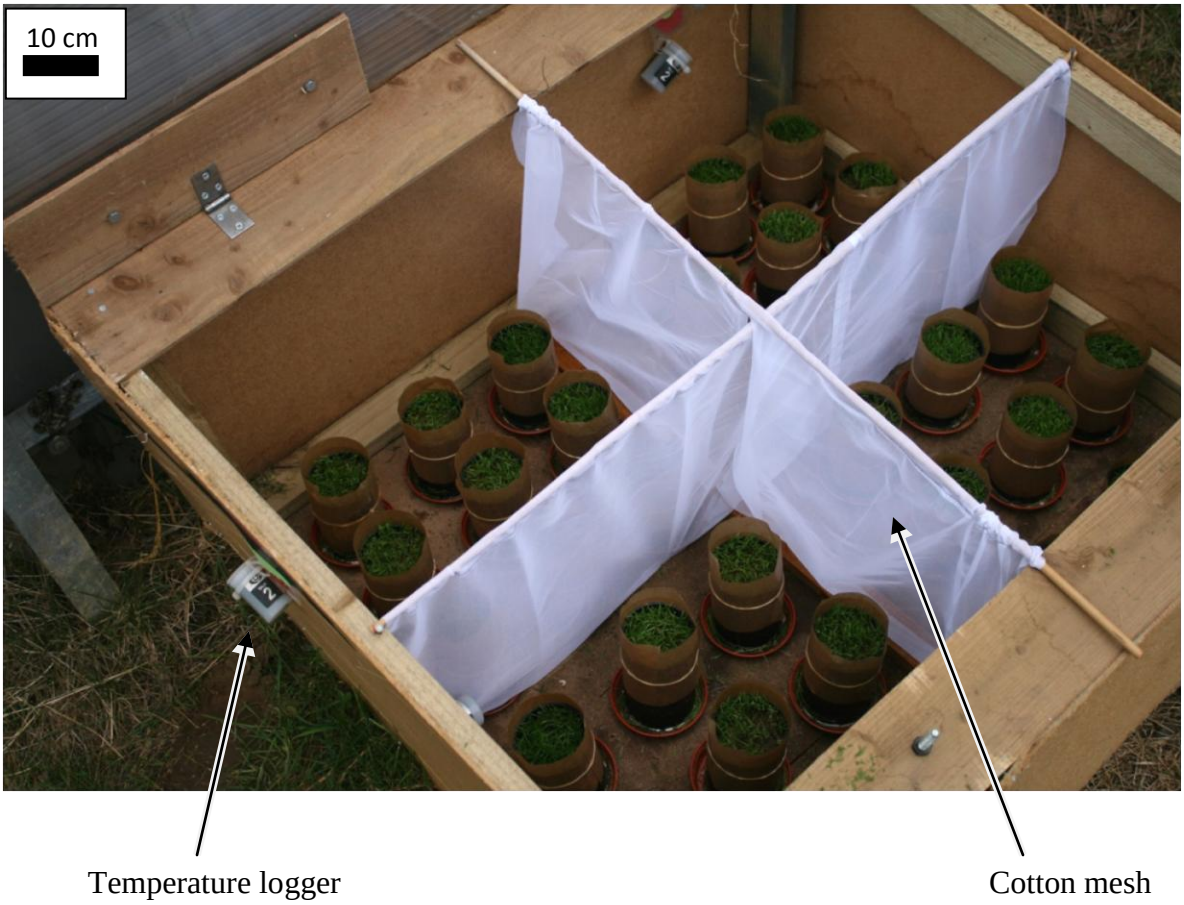


Figure 4.2 Cold frame arrangement containing treatments in a randomized block pattern

During the experiment the grass in each pot was cut to 15 mm every 2nd day using scissors, sterilized with ethanol between treatments. Treatments were watered with 25 ml (SDW) every 2nd day.

4.2.6 Disease assessment

Image analysis was used to record the incidence and severity of disease within the separate treatments. After initial inoculation, photos were taken of individual microcosms daily for the first week and then every 2 days thereafter; taken in plan view at a height of 40 cm

using a Canon EOS XTi camera (EFS lens, 18-55 mm). The photos were then subjected to analysis in ImageJ (ImageJ, Image Processing and Analysis in Java). In each image the total area inside the microcosm was measured as numbers of pixels, individual areas of disease were then isolated subjectively within the image and area determined as numbers of pixels. The areas of disease were then expressed as a percentage of the total microcosm area.

4.2.7 Subsequent sampling

Four randomly selected leaves were sampled from each of the four replicates containing the sprayed spore solution inoculums (Treatment 8) and placed into Petri dishes containing PDA (Oxoid) and 50 mg/l chloramphenicol (Fisher scientific). Samples were then incubated at 18^{o}C for 72 hours and inspected for growth of *M. nivale*. Viability of spores and the host plants susceptibility to disease were recorded. Samples of grass with evident mycelial growth were sub sampled after the experiment had finished and plated on PDA + chloramphenicol. The plates were left in an incubator at 18^{o}C until mycelia had grown sufficiently to identify morphologically. This was necessary to ascertain that the presence of any mycelium during the duration of the experiment was indeed *M. nivale* and not that of another plant pathogen.

4.2.8 Statistical analysis

All data were first checked for normality and homogenous variance (Anderson-Darling). One way analysis of variance was used to compare means (Mini-Tab). A post-hoc Tukey test was then applied.

4.3 Results

4.3.1 Enumeration of spores (*M. nivale*)

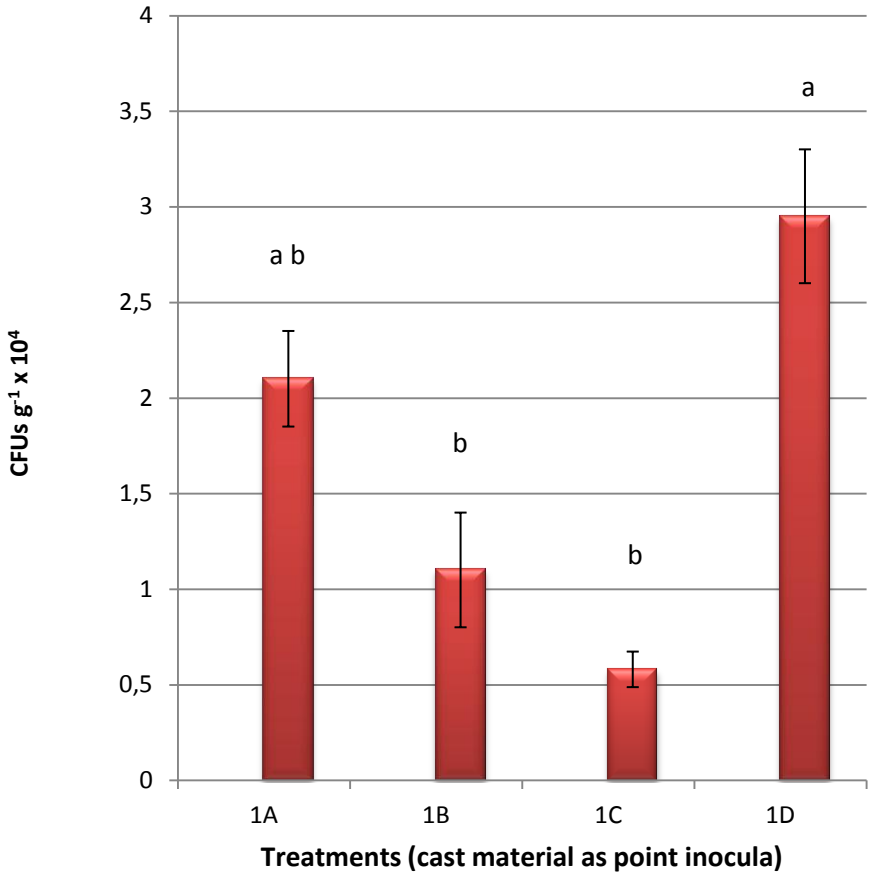


Figure 4.3 Number of *M. nivale* CFUs isolated in cast material post ingestion + SE. Letters denote homogenous groups at P = 0.05

CFU's isolated from all cast material were statistically analysed to show significant differences in the amount of CFU's present. Using one way ANOVA and a subsequent Tukey test, it was apparent that there were significant differences in the amount of CFU's

isolated in the cast material. Numbers of CFUs varied between replicates 6-fold ($P < 0.05$; Figure 4.3), and were of the order of 10^4 CFUs per gram.

Analysis of the cast material from the microcosm containing no *M. nivale* (control) showed little fungal contamination, typically 3-4 colonies per plate. The fungi were not identified morphologically and no evidence of *M. nivale* was seen on any of the control plates.

4.3.2 Infection results

Figure 4.4 Mycelial growth in Treatment D8 (spore suspension sprayed, $1.7X10^5$ CFUs/ml)

The manifestation of mycelium denoting the presence of *M. nivale* was only evident in three of the microcosms, two from Treatment 4 ($1.7x10^2$ CFU/ml) and one from Treatment 8 (sprayed inocula). With respect to Treatment 4, there was also great variation between these two instances (Figure 4.5).

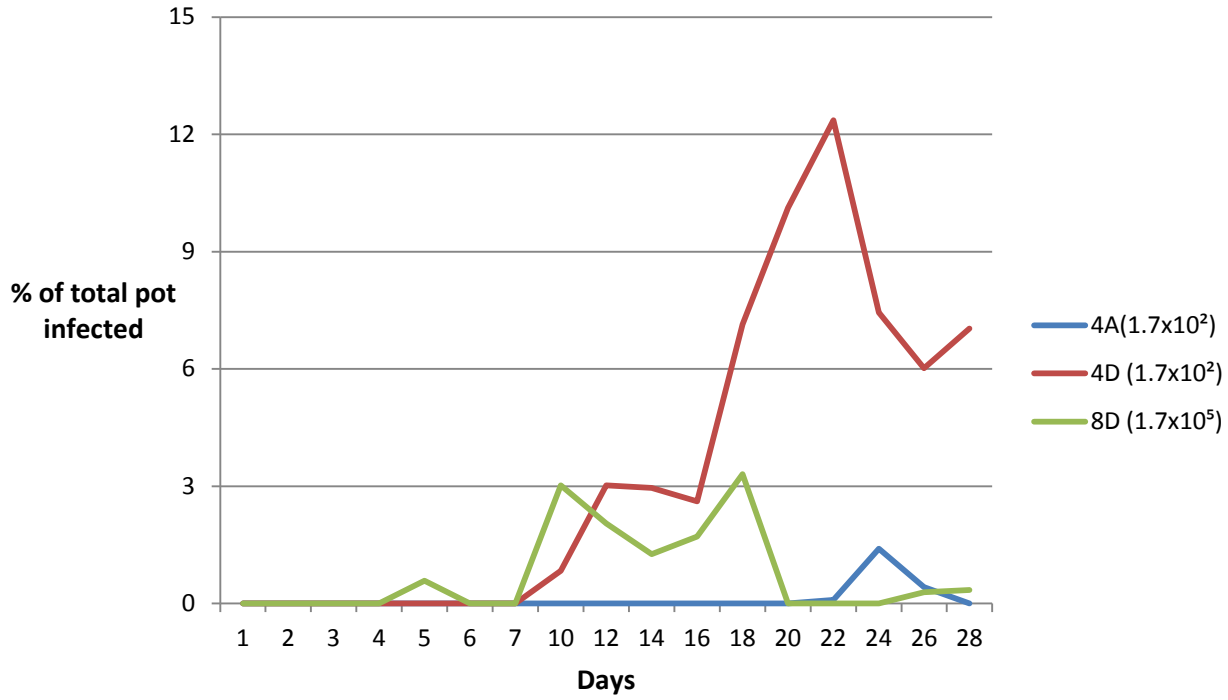


Figure 4.5 The mycelial dynamics in the three instances where growth was manifest (% of total area) of *M. nivale* in treatments 4A, 4D & 8D

Almost all mycelia presence within the three treatments began at a similar time, approximately one week after the experiment had started. Observations made while enumerating CFUs during soil dilution procedures also reported that CFU growth tended to be accessible after 5 days. The highest level of mycelia presence at any stage during the process of the experiment was found in Treatment 4D with a total microcosm infection of 12%, e.g. Treatment 8D shown in Figure 4.4. Very few leaves exhibited lesions or necrosis

of any form. Observations over the period of the experiment noted that mycelium appeared intermittently, maximum levels of mycelial presence (Treatment 4D) were evident after the 22 days, and then declined considerably.

When recording disease manifestation over the experimental period it was noted that the cast material inoculated into the centre of the microcosms had stayed intact and the cast material was still recognizable in its form.

4.3.3 Viability of *M. nivale* in Treatment 8

The leaves sampled one week post establishment from each of the four treatments inoculated with sprayed spore suspension (Treatment 8) showed evidence that *M. nivale* propagules were present within the treatment as exhibited by its recovery. Of the 16 leaves sampled on PDA + Chloramphenicol, 15 leaves exhibited mycelial growth identified as *M. nivale* (Figure 4.6),

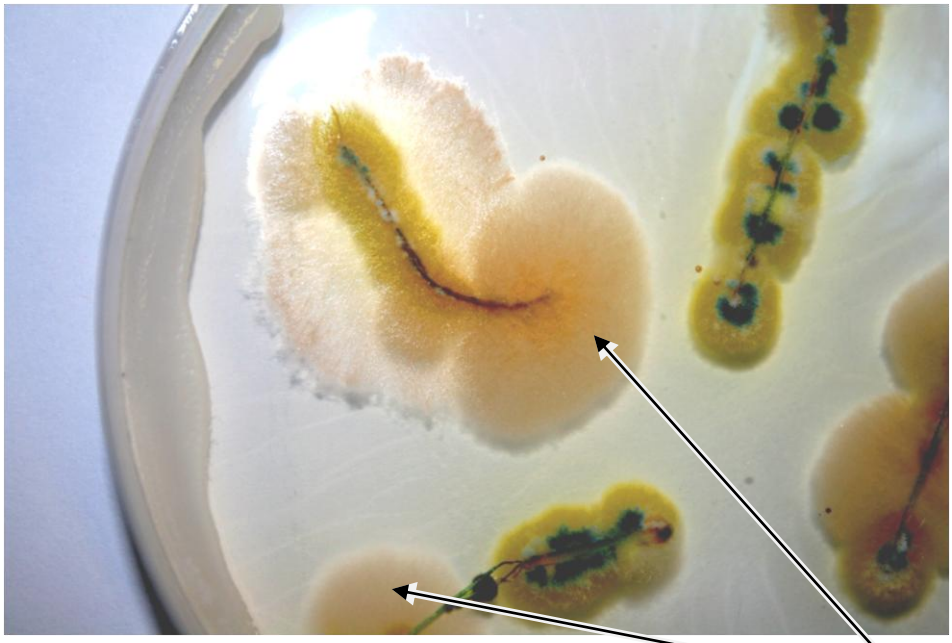


Figure 4.6 PDA plate + Chloramphenicol showing leaves infected with *M. nivale*

4.3.4 Pathogen identification

All samples of grass exhibiting mycelia growth after the duration of the experiment were also sampled on PDA + Chloramphenicol. All leaves sampled produced fungal growth attributed to *M. nivale* and were identified using the descriptive key found in Nelson *et al.* (1983). In all treatments no signs of other pathogenic diseases were observed.

4.3.5 Temperature

Mean temperature maxima and minima are shown in Figure 4.7. Maximal temperatures recorded by the loggers both in and outside the cold frame frequently exceeded 40^{o}C. For the period of the experiment, high mean temperatures found in and outside the cold frame were substantially higher than maximum air temperatures retrieved from the Met Office for the same period in the Bedfordshire area (Figure 4.7). Average high temperature over the 28 day experimental period from the Met office was 12.1^{o}C, as opposed to an average of 27.5^{o}C for the high temperatures recorded by the temperature loggers. Figure 4.7 includes parameters identifying the optimal high and low air temperatures (0 – 20^{o}C) required for disease development (Hsiang, 2007). Mean low temperatures were present inside the temperatures conducive to disease manifestation.

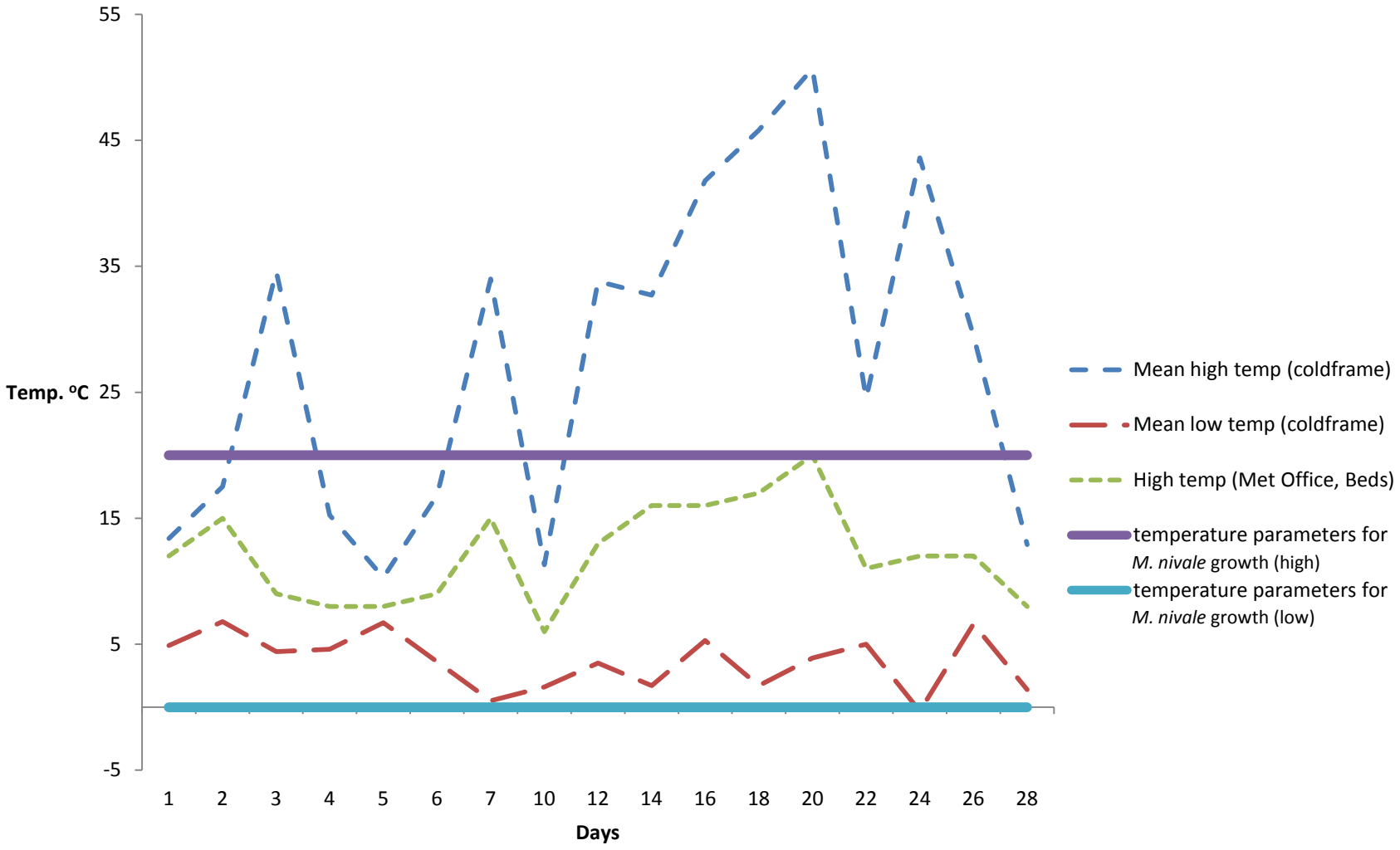


Figure 4.7 Maximum and minimum temperature profiles

4.4 Discussion

This study has clearly demonstrated that worm casts containing viable propagules of *M. nivale* had no effect on the incidence and severity of Fusarium patch in *Lolium perenne* compared to the control microcosms. With no precedent studies found pertaining to the dissemination of pathogenic propagules in cast material, various underlying factors could have contributed to the low amounts on infection in this study.

Mycelial development, considered a pre-requisite to infection was only observed in three instances and was limited to treatments inoculated with spore suspensions containing conidia in concentrations of circa. 1.7×10^2 CFU/ml (i.e. Treatments 4A & 4D) and 1.7×10^5 CFU/ml (Treatment 8A) respectively. Comparison of the treatments with mycelia development showed no distinct differences in mycelia growth dependent on inoculum loads present. This was reflected in two of the treatments exhibiting mycelia growth (4A, 4D) from point inoculums considerably lower in concentrations than those of the sprayed Treatment (8D). Viability of the spores was ascertained by leaf sampling from treatments inoculated with the sprayed spore suspension; therefore it is assumed that the low abundance of mycelia growth, coupled with very limited necrosis in the leaves was a direct result of the pathogen not manifesting itself.

Cast material proffered by the earthworms as a source of point inoculum for the purpose of this experiment contained CFU counts of between $0.5 – 3 \times 10^4$/CFU g^{-1}. Concentrations of CFUs in the cast material were distinctly lower than those isolated in the previous experiment, which can be explained by the constraints on spore re-isolation post ingestion

outlined in Chapter 3. The cast matter exhibiting variation in the numbers of CFUs enumerated enabled the hypothesis to be tested using four independent replicates containing various inoculum loads. Previous work on earthworms transmitting viable propagules of fungi through earthworm casts have focused on critical inoculum loads present within cast material affecting infectivity (Reddell and Spain, 1991). In this study critical inoculum loads were not identified as no disease was manifest. Although disease was absent, spore solutions sprayed onto the entirety of the plant in significantly higher CFU concentrations would however negate this argument. Previous studies have shown that earthworm casts typically contain high levels of nutrients, which in turn support a varied microbial community and the production of gram-negative bacteria (Edwards and Arancon, 2004; Clapperton, *et al*., 2001). This organically rich material has been shown to suppress disease, and research has indicated that some species of *Fusarium* had poor survival rates in worm casts where microbial activity was high (Toyota and Kimura, 1994). Had this study utilized cast material collected from a non sterile/*Microdochium* rich environment, these views could have provided evidence as to why no infection was discernible from the cast treatments. However, the casts were collected from earthworms fed on steam sterilized soil; therefore microbial activity would have been limited due to the sterilisation processes involved.

From this study there is no evidence to suggest that latent infectivity is either present or greater in the presence of earthworm casts containing viable spores of *M. nivale* than the inocula in the form of spore solutions. It is not possible to determine the significance

infected worm casts have on disease manifestation in amenity turf other than the lack of infection present.

The absence of Fusarium patch in any of the treatments can be attributed to a number of considerations all of which have the propensity to inhibit disease manifestation. Only small amounts of isolated mycelial growth were evident, culminating in a maximum of 12% infection at any one time. The appearance of intermittent growth suggests that apart from the host plants possible resistance to disease, high temperatures within the cold frame reached levels inhibiting further growth. According to Hudec and Muchova (2010), *M. nivale* averages greater mycelial growth at 15^{o}C than 25^{o}C, almost a 50% increase at the cooler temperature over a period of 5 days. This is reiterated by Vigier, *et al.* (1997) and Mann and Newell (2005) who placed great importance on the role of temperature and fluctuating environmental conditions coinciding with the incidence and severity of *Fusarium spp*. and importantly, *M. nivale*. Low level temperatures recorded during the experiment were considered optimal for disease development with *M. nivale* more active at temperatures slightly above freezing (Mahuku, *et al*., 1998), in contrast, temperatures above 20-25^{o}C are not considered conducive to disease development (Arsvoll, 1975; Okuyama, *et al.*, 1998). This data suggests that once infection had started due to optimal low temperature, the high temperatures recorded by the loggers may have had an inhibitive effect, the process of disease development subjected to a stop start reaction, whereby temperatures were efficacious at certain points throughout the day, and at other times unfavourable. Viability of the spores present on the leaves was assured, reflected in the

leaf assays during the experimental period; therefore it can be assumed that spore germination was awaiting the correct environment or host.

Environmental conditions can also have adverse effects on spore survival. Toyota and Kimura (1994) showed that collected casts containing viable spores of *Fusarium oxysporum*, when incubated at 28^{o}C, resulted in a decline in spore numbers. Although looking primarily at *M. nivale*, speculative comparisons can be made with other species of *Fusarium* due to similar physiological traits (Hsiang, 2007). Temperatures registered by the probes during the experimental process showed highs exceeding 50^{o}C with mean temperatures considerably higher than those obtained from the Met Office in Bedfordshire. This indicates that the cold frame used induced higher temperatures due to its position in the test site; the cold frame was placed in close proximity to a metal fence and although predominately in the shade, the fence could have contributed to the higher temperatures. Exposure to direct sunlight periodically throughout the day could induce a glasshouse effect, accentuated by the Perspex lid of the cold frame. Koiyumi (1993 cited in Tronsmo, *et al.*, 2001) investigated the survival of *M. nivale* conidia in soil of differing temperatures, and noted that conidia present in soils subjected to 15 and 25^{o}C temperatures, remained viable for 30 and 10 days respectively. The high temperatures experienced throughout the current experiment could imply that spore survival in the cast material was restricted, reducing viability and yielding little infection throughout the process. During the duration of the experiment, samples of soil were not tested for evidence of *M. nivale*, which could be considered an error in the sampling strategy.

Aside from the environmental conditions influencing disease manifestation, there are also contradicting views as to the susceptibility of rye grass to *M. nivale* compared with other grass types. Trials conducted by the STRI indicated that perennial rye grass is not as susceptible to *M. nivale* as some species of *Festuca* or *Agrostis* (Mann, Personal Communication, 2012), whilst Raikes, *et al.* (1996) and Gange and Case (2003) viewed *Lolium perenne* to be moderately vulnerable to *M. nivale*, Pronczuk and Messyasz (1991 cited in Tronsmo, *et al.*, 2001) demonstrated in trials with *Lolium perenne* that inoculation with *M. nivale* conidia resulted in no infection. However, inoculation with *M. nivale* mycelia initiated severe symptoms; this research could imply that only certain types of inocula have capabilities affecting both the incidence and severity of disease. Of the three types of inocula responsible for infection, (conidia, mycelia and ascospores), views range as to which is the primary source initiating outbreaks of disease (Tronsmo, *et al*., 2001). According to British seed houses, the cultivar of rye grass used (Romance) has a high disease resistance scoring against *Laetisaria fuciformis* (Red thread) and *Sclerotinia homoeocarpa* (Dollar spot). Unfortunately susceptibility to Fusarium patch, the disease caused by *M. nivale*, is not recorded due to difficulty in inoculating the disease in controlled conditions (Hendy, British Seed Houses, Personal Communication, 2012). With limited information regarding the susceptibility of amenity turf to Fusarium; a cultivar not susceptible to this disease could have inadvertently been prescribed for this experiment. Indeed, further investigation into whether this is an inherently resistant cultivar may be worthwhile.

4.4.1 Conclusions

Due to the lack of data regarding infection, the findings indicate that the research hypothesis **'Earthworm casts containing propagules of *Microdochium nivale* when introduced into turf will lead to manifestations of Fusarium patch'** cannot be accepted. The amount of data obtained has led this study to question the many variable factors associated with disease development. What can be ascertained from this research, rather obviously, is that there is no evidence for increased liability of infection where conditions are not conducive to disease development. From this statement we can assume that earthworms do not necessarily pose a threat to rye grass swards with regards to the dissemination of infectious propagules of *M. nivale*. What is pertinent to note, is that disease did not manifest itself regardless of whether the spores of *M. nivale* had passed through the digestive system of an earthworm or not, and in these circumstances inoculum derived from worm casts is no more potent than the point inoculum derived from the original source. It can therefore be postulated that the spores passage through an earthworm does not result in an increase in pathogenicity or infectivity, at least in the specific environmental conditions in this study.

4.5 Further experimental research

Due to the lack of sample data arising from this experiment, it was deemed necessary that, in order to more accurately answer the research hypothesis outlined in the introduction, further experimental research would be appropriate. In this vein, the experiment could be reproduced, addressing the underlying factors contributing to the lack of data originally provided. Having assessed the virulence of *M. nivale* through leaf sub sampling in PDA it

was reasonable to assume that the method of inoculation and indeed the inoculants were suitable to initiate infection. For the repeat experiment, temperature and grass species would be the variable factors changed in incorporating a methodology which would better answer the research aim. Couch (1973 cited in Gange and Case, 2003) lists 85 grass hosts susceptible to *M. nivale*. Replacing *Lolium perenne* with a species of bent for e.g. *Agrostis stolonifera* commonly used for amenity turf grass predominantly in a golf course environment would provide a greater understanding of disease susceptibility with mainstream grass species used in fine turf. The use of a growth cabinet or similar mechanism to control environmental conditions would enable temperatures to be regulated and set within the parameters identified as optimal for disease advancement. The limited sample data from the *L. perenne* experiment has provided the opportunity to assess all the variable factors present during the study and make the necessary changes in order to better understand the environmental criteria benefiting pathogen progression.

Chapter 5: The effects of *Lumbricus terrestris* on the dispersal of *Microdochium nivale* in *Agrostis stolonifera* turf

5.1 Introduction

The previous two chapters of this thesis have investigated spore viability of *M. nivale* post ingestion and the ramifications this may have on the infectivity of propagules found within the earthworm's faecal matter. It has been confirmed that spores do survive the digestion process, although the latent infectivity of worm casts was not robustly established. The outcome of these studies has identified the need for different modes of pathogen dispersal to be considered, to understand the potential roles that earthworms play in disease dissemination. In turf grass limited information is apparently available regarding the dissemination of plant pathogens by means other than consumption. External carriage, whereby propagule attaches to the external wall of the earthworm must be considered a method by which pathogens can be transported within the soil substrate. Agrios (1980) investigated the roles of soil biota in pathogen dispersal via external contamination, although this research was focused more on the role of insects and nematodes as vectors of fungal pathogens as opposed to annelids. Toyota and Kimura (1994), studying pathogen survival through the earthworm *Pheretima spp*. observed that spores of *Fusarium oxysporum* were evident in the burrows leading away from sources of inoculum; however, they reiterated that general pathogen dissemination was a result of ingestion and ejection. Due to the emphasis placed on consumption of microorganisms regarding pathogen dissemination, very little information is available on the dispersal of these organisms via earthworm movement. Anecic and endogeic earthworms are the predominant movers through semi-permanent vertical and horizontal burrows. These species are considered

more efficacious in the movement of beneficial and detrimental microorganisms purely due to the greater distances these worms can travel as opposed to epigeic forms. This aim of this investigation was to examine the effects that the presence of anecic earthworms have on the dissemination of *M. nivale* in turf grass. Emphasis was placed on whether the inclusion of earthworms accelerated disease development. Due to the anecic earthworms extensive movement through the soil matrix and their close proximity to large amounts of microorganisms and fungi, a substantial part of the earthworms diet, it was postulated that propagules would be dispersed wherever the earthworm travelled, through a consolidation of taxis, and re-establish in a new environment. This was examined by testing the following hypothesis:

'The presence of earthworms will increase the rate of dispersal of *M. nivale* in amenity grass more so than in the absence of earthworms'

To test this, a pot experiment was conducted using *Agrostis stolonifera* turf systems point-inoculated with spore solutions of *M. nivale* in the presence and absence of anecic earthworms.

5.2 Material and methods

5.2.1 *Microdochium nivale*

A culture of sporodochia producing *M. nivale var. nivale* (Strain 1815) on PDA was acquired from The Food and Environmental Agency (FERA, London, England). A spore solution was prepared using the same methods described in the Chapter 3, section 2.1. The spore solution was adjusted appropriately to provide 8 x 10^5 spores/ml, confirmed by haemocytometer- based enumeration.

5.2.2 Earthworms

Adult earthworms of the species *Lumbricus terrestris* were used for this experiment. The earthworms were obtained from Bleak Hall sports shop (Kempston, England) and were identified using the OPAL key to common British earthworms (Jones and Lowe, 2011). Earthworms were sanitized using the same method described in Chapter 3 (section 2.2), ensuring all gut contents were removed and that no residue was attached to the external wall of the earthworms. This was achieved by gentle rinsing under STW. Sanitized earthworms were stored in Petri dishes until needed.

5.2.3 Amenity grass microcosms and soil

Agrostis stolonifera L. Cv. Providence (British Seed Houses, Bristol, England) was seeded into polypropylene pots (microcosms) measuring 230 x 180 x 60 mm (Figure 5.1) containing a sandy-loam soil classified as 3 mm screened and sterilised with a particle analysis of 75-18-7 sand, silt and clay (Boughton loam, Telford, Northampton). The seeding rate was equivalent to 15-20 g/m^2. The turf microcosms were then placed into a

greenhouse and misted frequently with STW until germination had been achieved. The microcosms were watered as required to facilitate optimum growth and the height of the sward was maintained to between 10-15 mm. The microcosms were conditioned in the greenhouse for a period of 4 weeks prior to the experiment.

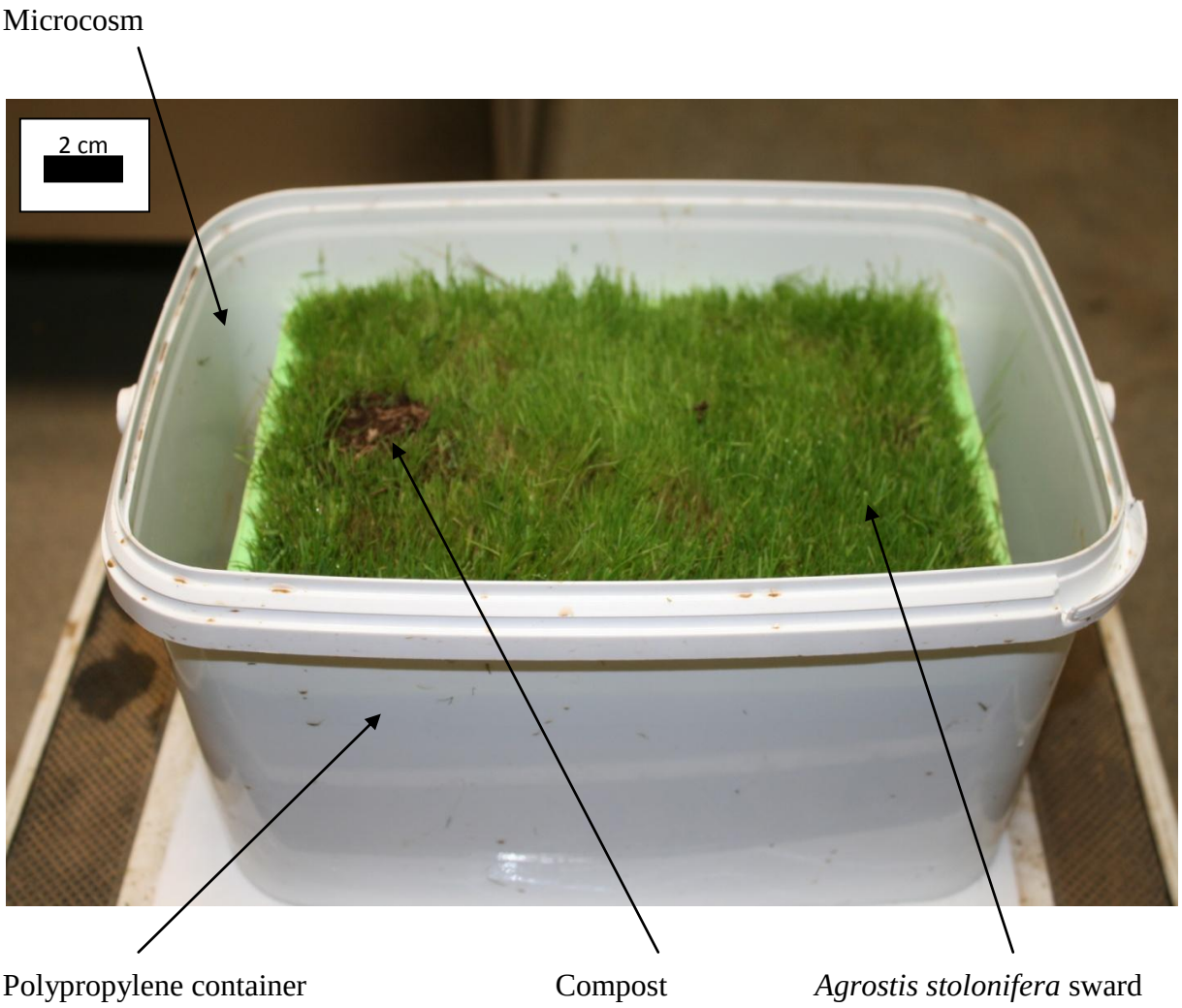


Figure 5.1 Overview of turf microcosm (*Agrostis stolonifera*) contained in a polypropylene perimeter

5.2.4 Points of inocula

Westland bulb planting compost (Westland Horticulture Ltd, Huntingdon, England) was used as the inoculum source for the purpose of this experiment. The compost was macroscopically cleared of larger debris and then steam sterilised at 121^{o} C for 30 minutes. The compost was then stored in air tight containers at 4^{o} C until utilised.

5.2.5 Experimental design

The experiment consisted of five treatments: (1) Microcosm containing compost spiked with *M. nivale*; (2) Microcosm containing compost free from *M. nivale;* (3) Microcosm containing compost spiked with *M. nivale* plus earthworms (x2); (4) Microcosm containing earthworms (x2); (5) Microcosm containing compost free from *M. nivale* plus earthworms (x2) (Figure 5.2).

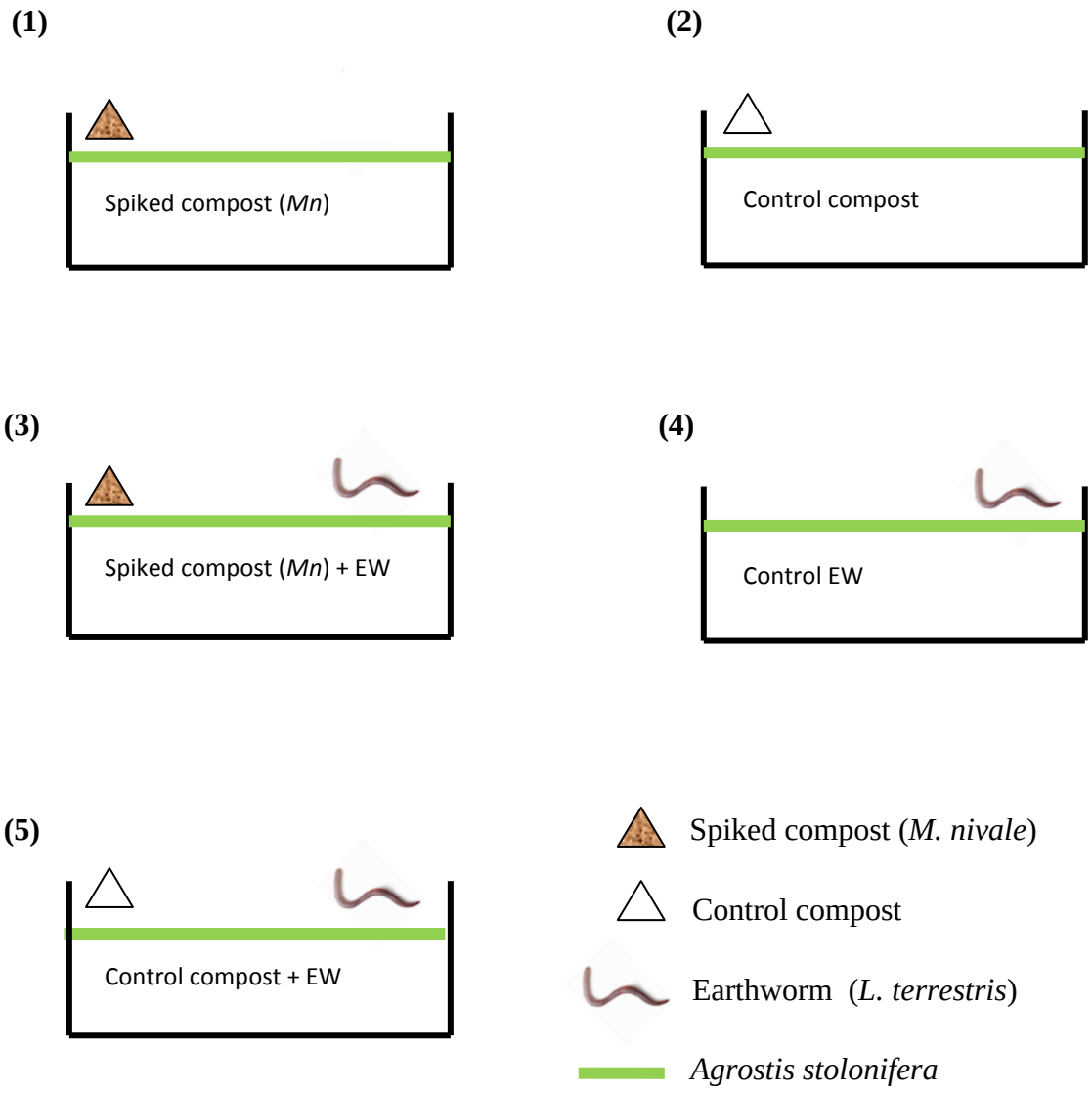


Figure 5.2 Experimental design to investigate the dissemination of *M. nivale* in the presence and absence of earthworms.

Four independent replicates of each treatment were established (denoted A-D), arranged in a randomised block design. Each microcosm was placed within a polypropylene container to restrict the migration of earthworms present in the microcosms.

5.2.6 Experimental procedure

In treatments containing earthworms, sanitized voided earthworms (x2) were transferred to each microcosm and placed randomly on the surface of the sward. Aliquots (5 g) of steam sterilised compost was placed at the extreme left of each microcosm; disease dispersal and manifestation could be better observed as the disease progressed outwards. Where applicable, the compost was then inoculated with 1 ml spore suspension containing 8×10^5 spores/ml.

The microcosms were then placed in an incubator (Snijder, Tilburg, Netherlands) with a 16/8 hours light dark cycle corresponding with temperatures of $20/12^{o}C$ light/dark respectively for a period of 14 days. The application of STW into the external container in which the microcosms were placed ensured the swards maintained appropriate moisture levels. Grass was maintained to an approximate height of between 10-15 mm and the microcosms were misted twice daily with STW to maintain leaf wetness and encourage disease manifestation.

5.2.7 Disease assessment and sampling strategy

Throughout the duration of the experiment photographs were taken daily in plan view at a height of 60 cm using a Canon EOS XTi camera (EFS lens, 18-55 mm). Image analysis was then performed using the same procedure described in Chapter 4, section 2.6. Diseased areas were expressed as a percentage of the total microcosm surface area (0.04 m^2).

Using a stratified random sampling strategy in three equally sized sectors across the microcosm area (Figure 5.3), leaves were sampled from each microcosm on the 4th, 8th and 12th days after establishment. From each point in the segment, the three nearest leaves were removed and plated onto PDA, (Oxoid) + Chloramphenicol (Fisher scientific). Subsequent mycelia growth was identified using the descriptive key found in Nelson, *et al.* (1983).

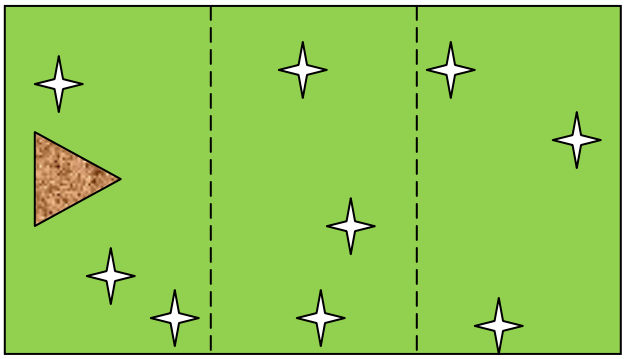


Figure 5.3 Plan view of microcosm separated into three equal sectors with examples of stratified randomised sampling points.

Plates exhibiting mycelia growth morphologically similar to *M. nivale* were sub-sampled onto fresh PDA plates and subjected to daylight to encourage colony formation and pigmentation. Comparisons were made to descriptive and pictorial keys (Nelson *et al.* 1983) and identified through the authors experience as *M. nivale*. The absence of spores in the plates ensured identification was primarily through the traits of mycelia growth and

colour, the latter providing evidence in the form of white/salmon pink flocculated mycelium growth characteristic of *nivale*.

5.2.8 Worm weight and cast analysis

After the experimental period was complete all casts were collected from the microcosms and independently tested for the presence of *M. nivale*. Enumeration of CFU's from the cast material was determined using the same procedure outlined in Chapter 3. Diluted samples were spread onto PDA plates containing 50 mg Chloramphenicol to inhibit bacterial growth and enumeration of spores was represented on a $CFUg^{-1}$ dry matter basis. Where applicable, evidence of *M. nivale* growth was sub-sampled onto fresh PDA plates to assist with identification.

On conclusion of the experiment recovered earthworms were left for 2 days to allow for evacuation of the gut contents and then re-weighed.

5.2.9 Statistical analysis

All statistical analysis was performed using Minitab 16 and Statistica, All data were first checked for normality and homogenous variance. Proportion of turf area infected by Fusarium patch was analysed using repeated measures analysis of variance. Analysis of worm weights was performed using one way analysis of variance; a post-hoc Tukey test was applied to identify homogenous groups.

5.3 Results

5.3.1 Controls

M. nivale was not detected in any instances of sampled leaves derived from the control treatments. Observations further noted that no evidence of other fungal pathogens were present in the control microcosms.

5.3.2 Infection

Fusarium was evident in the turf as mycelia and in the form of necrosis of the leaves. Mycelium was typically restricted to the outside areas of the infection as the disease progressed throughout the microcosm. The disease was manifest with leaves exhibiting a distinct water- soaked appearance. Pigmentation of the leaves was typically red-brown in colour, consistent with infestations of Fusarium patch in *Agrostis* grass (Figure 5.4).

Figure 5.4 (A) Example of manifestation of Fusarium Patch in treatment (1A)

(B) Close up of Fusarium patch with water soaked appearance typical in *Agrostis*

5.3.3 Disease assessment and microcosm sampling

By day 4 only one microcosm from Treatment 1 had pathogen presence on the leaves and this was found in Sector 1. Whilst by Day 8, *M. nivale* was present in the first sector for all Treatments, in the second Sector propagules of *M. nivale* were more evident in Treatment 3 (Figure 5.5). Propagule assessment for Day 12 showed that 3 of the 4 replicates from Treatment 1 had progressed into Sector 2; however, only one microcosm exhibited pathogen progression into Sector 3. This is in contrast to the Treatment 3 as all Sectors showed evidence of *M. nivale.*

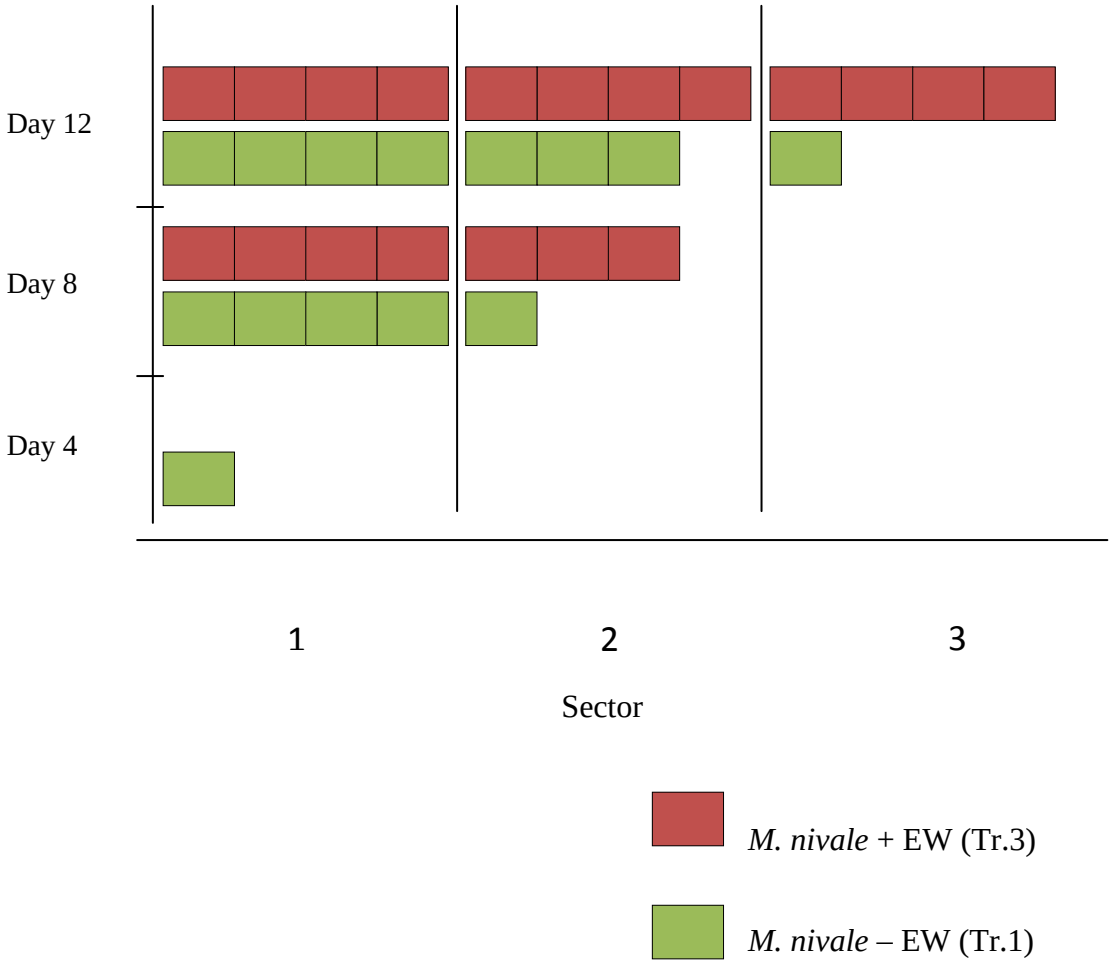


Figure 5.5 A schematic plan of propagule dispersal in the presence and absence of earthworms assayed through leaf sampling. Number of replicates containing *M. nivale* (out of four), denoted by square symbol.

5.3.4 Image analysis and infection results

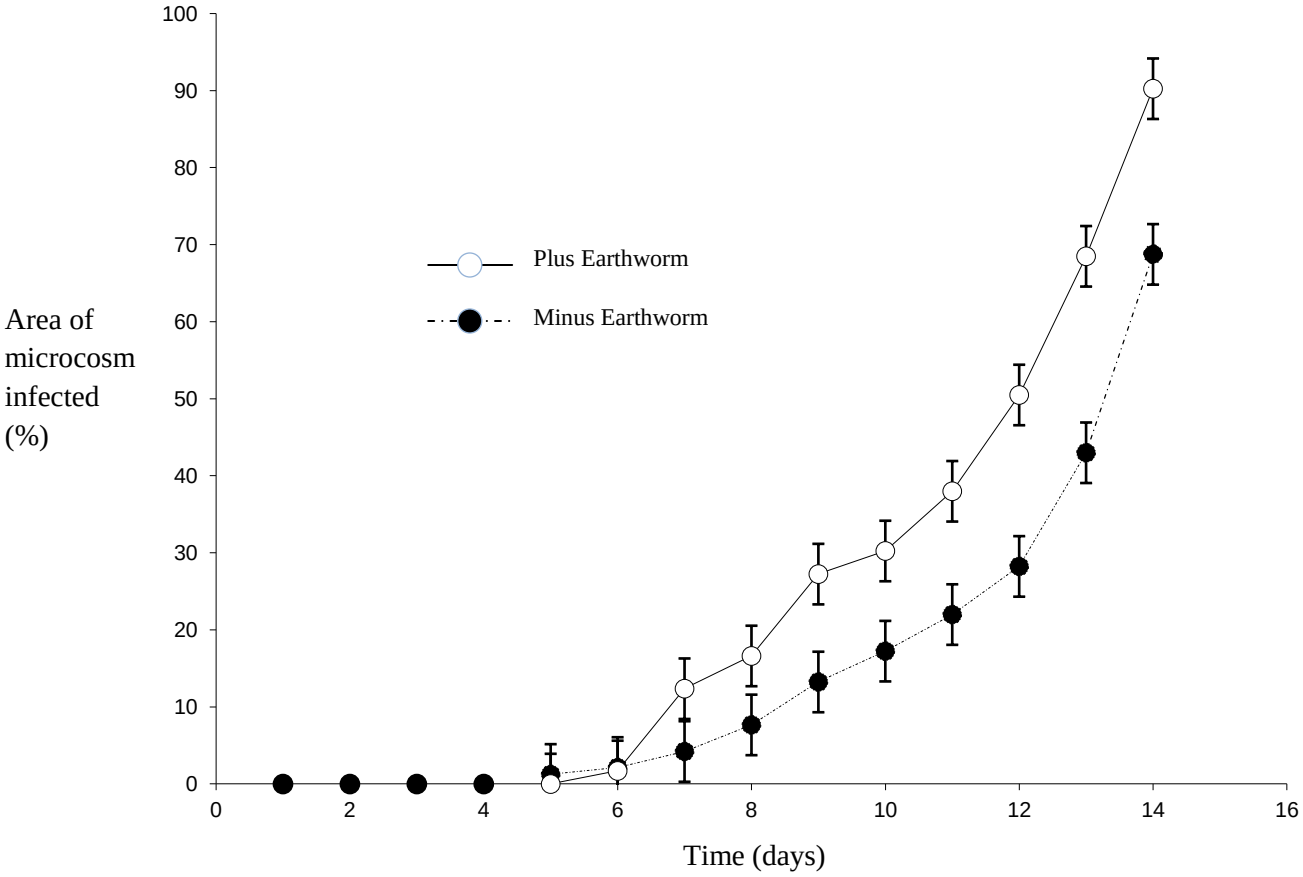


Figure 5.6 Proportion of turf affected by *M. nivale* over time in presence and absence of earthworms. Points show means (n=4). Bows indicate standard error of the means.

Disease progression commenced from Day 5 for the microcosms containing no earthworm and Day 6 for the microcosms containing spiked compost plus an earthworm (Figure 5.6). The disease progression rate (mean) from the start of infection to the end of the experiment was 6% per day for the microcosms containing spiked compost and no earthworm, and 9 % per day for the microcosm containing spiked compost and the addition of an earthworm.

Given this development pattern, RM-ANOVA was applied incorporating from Day 5. There was a highly significant time effect on disease progression ($F_{9,54}$ = 82, P < 0.001).However, there was also a significant time x treatment interaction, whereby the manifestation and rate of disease was significantly increased by the presence of earthworms as opposed to the absence of earthworms ($F_{9,54}$ = 2.7, P = 0.01). Analysis of the data on the overall treatment effects that the presence and absence of earthworms had on disease manifestation showed marginal significance ($F_{1,54}$ = 4.31, P = 0.08), indicating that disease progression was different, dependent on treatment.

5.3.5. Worm weights and cast analysis

All 24 earthworms were recovered from the microcosms. After gut voidance and weighing it was apparent that there was no detectable weight gain in earthworms dependent on Treatments (P > 0.05). The mean weight gain of earthworms from the microcosm containing *M. nivale* was 15.87 ± 2.8 mg as opposed to 11.37 ± 3.78 mg for the earthworm plus control compost. The control earthworm had a mean weight gain of 11 ± 3.92 mg.

5.3.6 Casts analysis

Only small numbers of worm casts were found on the surface of the microcosms. In Treatment 3 (*Mn*+EW) a total of 9 casts were recovered from all replicates, 9 casts were also recovered for all replicates in Treatment 4 (EW) and 10 casts were recovered in Treatment 5(Control compost + EW). The average worm cast weighed 0.85 mg. *M. nivale* was detected in all casts found in treatment 3. However, establishing numbers of CFUs of *M. nivale* was not possible due to the presence of other fungi on the plates

obscuring enumeration. Sub-sampling ensured colonies were identified and attributed to *M. nivale*. No background levels of *M. nivale* were found in any of the casts from the control worms.

5.4 Discussion

5.4.1 Disease manifestation

The results have clearly demonstrated that the presence of earthworms had a greater effect on the rate of development and severity of Fusarium patch in *Agrostis stolonifera* than if earthworms were not present.

Data derived from the image analysis showed a highly significant time by treatment interaction ($P = 0.01$) which clearly showed that the addition of earthworms contributed to an increase in the rate and progression of Fusarium patch in amenity sports turf. The experiment was designed to determine how earthworms affect the dissemination of propagules within turfgrass, leading to manifestations of disease. The results have indicated that different processes may be involved in this dispersal.

The first process by which propagule dispersal and subsequent disease manifestation can be explained is the survival of viable propagules in the earthworms faecal matter. Chapter 4 hypothesised that propagules of *M. nivale* found in the faecal matter had the propensity to infect turf; this was not established due to constraints within that experiment. However, both environmental and host conditions were significantly different during the present

study and therefore casts were not collected as they may have had an efficacious effect on disease manifestation. The data concluded that cast material recovered from Treatment 3 contained propagules of *M. nivale*. This illustrates conclusively that first; an earthworm consumed spores of *M. nivale* and is consistent with the findings in both Chapters 3 and 4. Second, as all earthworms were recovered from their respective microcosms the earthworms exposure to *M. nivale* had no impact on mortality rates which can be associated with earthworms feeding on mycotoxin-producing fungi (Edwards and Fletcher, 1988; Wolfarth, *et al*., 2011). Although question marks have been raised regarding *M. nivale* and its ability to produce mycotoxins, particularly trichothecene which are hazardous to both animals and humans, *M. nivale* has been known to produce nivalenol, a toxin capable of contaminating food stuffs leading to substantial yield losses (Tronsmo, *et al*., 2001). Observations that anecic earthworms were not affected by consuming such fungi, albeit toxin producing or not, can better elucidate the need for identifying the role earthworms play in propagule dispersal. Particularly, since many fungal species including *M. nivale* have been shown to be preferred by certain earthworm species in food selection tests (Bonkowski, *et al*., 2000).

What can be ascertained from this study is that these propagules were not subjected to the higher temperatures recorded in the previous experiment (Chapter 4, section 3.5) which may have inhibited their viability. The temperature parameters were such that spore survival germination could be achieved when subjected to appropriate environmental conditions (Koiyumi, 1993 cited in Tronsmo, *et al*., 2001), therefore this mode of taxis cannot be excluded when considering all possibilities.

The other process involved in propagule dispersal is through external carriage. The schematic representation detailing propagule presence through leaf sampling provided evidence that propagules were dispersed more rapidly in the microcosms containing spiked compost and earthworms compared to the control microcosms. An explanation of this progression could be as a result of external contamination, whereby the movement of fungal propagules was accentuated by the soil fauna present. Of all the ecological groups of earthworms, anecics have the greatest dispersal potential for pathogenic fungi due to their burrowing and feeding traits and the greater distances that anecic earthworms can travel compared with endogeics and epigeics spp. (Friberg, *et al*., 2005). This study emphasised those traits whereby in all replicates containing earthworms, presence of propagules were evident in the furthest sectors away from the original inoculation points; less propagules were sampled in the final sectors in the absence of earthworms. This would imply that infective propagules were either carried externally by the earthworm during feeding, as reported by Toyota and Kimura (1994), or, consumed and ejected in the faecal matter, the cast acting as a contagion or secondary inoculum point.

The views that earthworms help with disease suppression assumes that either pathogenic spores do not survive the digestion process; inhibiting further germination. Or, that earthworm casting are microbially rich, especially high in nitrate levels which contribute significantly to changes in pH, microbial activity and the availability of micronutrients, all of these mechanisms helping in decreasing the severity of Fusarium diseases (Elmer, 2009). The findings of this study are however in contrast to views that augmenting

earthworms into soils heavily infested with plant pathogens can return soil productivity and decrease pathogenic fungal populations. It was not ascertained how many spores survived the digestion process as CFU enumeration was not possible. Data from Chapters 3 and 4 saw significant decreases in the concentration of CFUs in the worms faecal matter compared to the original inoculum point. It was not however apparent how many spores were destroyed or absent away from the sampling points through earthworm interaction, this could certainly be the case in this present study. It was clear from the data obtained from this experiment that the presence of earthworms had no inhibitory effect on the progression of propagules and the manifestation of disease, the inclusion of earthworms did at no stage contribute to disease suppression. In fact, quite the opposite was confirmed with the presence of earthworms significantly accelerating disease development. As reiterated previously in this discussion, the experimental design did not manage to differentiate between the methods of dispersal. Further experimental work in developing a design which could better elucidate the mechanisms involved in propagule dissemination is required. Cast infectivity could be ascertained by repeating the experiment in Chapter 4, incorporating the most efficacious environmental conditions available to aid germination of viable spores found in the faecal matter. To provide quantitative evidence that earthworms were carrying spores externally, a similar turf microcosm experiment could be repeated, whereby earthworms were systematically recovered and washed to dislodge any spores adhered to the cell wall. The CFUs could then be enumerated in the washings.

5.4.2 Conclusions and further work

The findings indicate that the data obtained was able to support and accept the hypothesis tested that '**The presence of earthworms will increase the rate of dispersal of *M. nivale* in amenity grass more so than in the absence of earthworms'**

This experiment has provided conclusive evidence that the plant pathogen *M. nivale* can be widely disseminated through earthworm activity. More importantly, in contrast to many studies, *Lumbricus terrestris* appears to have no inhibitory effects on the progression and manifestation of disease. Although the exact dispersal mechanisms were not identified, this study provides important information pertaining to the future management of earthworms in amenity sports turf. Selective fungal consumption by soil biota could significantly increase manifestations of disease in amenity sports turf. A management program for earthworm control based on these results would not momentarily be appropriate until further research is available regarding the pathogenicity of spiked cast material. Casts being the major management concern for sports surface managers. However, importantly, substantial evidence has been acquired that proves earthworms do not suppress *M. nivale* in *Agrostis* turf. Therefore, in areas of fine turf where the advantageous actions of earthworms is neither required nor deemed beneficial due to the greenkeepers care of such areas e.g. fertilising, top dressing etc. the management and control of earthworms should be a serious consideration, especially when environmental conditions are conducive to both casting activity and disease advancement.

Chapter 6: Synthesis, conclusions and further work

6.1 Introduction

Acceptable levels of disease on areas of fine turf are such that playability and the aesthetics are not affected. Participants in sports utilising fine turf rarely identify disease manifestations and have little knowledge as to the potential severity of disease infestations in turf. Sports surface professionals primary concern regarding disease manifestation is the homogenous health of the swards, therefore the use of chemical control is often overused in achieving the required results. Fungicide applications to treat disease incidence is an expensive solution, especially when a proper understanding of the intrinsic factors contributing to disease manifestation could alleviate such instances. The general perception of earthworms is that they are beneficial to their surrounding environment; this thesis has attempted to dispel this view in as much that earthworms can be detrimental to areas of fine turf in the movement of saprophytic plant propagules.

The advantages and disadvantages of earthworms upon the soil matrix have been well documented. The literature review provided extensive views relating to the many different functions provided by annelids within the various ecosystems in which they inhabit. The literature review further provided a description for the context of the study whilst identifying a theoretical framework on which to base this thesis.

The benefits of high earthworm populations in the soil matrix are many, the negative effects from high earthworm population, especially with concern to amenity sports surfaces are largely forgotten. Earthworm castings have long been considered a critical problem for

the sports surface managers. Pristine playing surfaces are considered a pre-requisite, especially in the context of golf courses; and casts, which affect playability and maintenance of such surfaces, are detriment to achieving these results.

The aim of this thesis was to provide evidence that aside from problem casting associated with earthworms, there are indeed larger issues that may need to be addressed, specifically, the movement of microbes within the soil matrix. Earthworms through their burrowing traits and feeding habits move large quantities of soil microbes, many of these microbes (e.g. mycorrhizae) play an important role in nutrient uptake in plants and such movement by soil fauna is recognized as an important function, given that various microbes have the inability to travel extensively through independent means (Hornby, 1990). However, pathogenic microbes also transported by soil biota can have major detrimental effects on the health of plants in the form of disease, in the context of amenity sports surfaces, resulting in issues affecting playability and aesthetics.

The experiment conducted as part of Chapter 3 concluded that spores of *M. nivale* did pass through the digestive system intact, even though the gut has been identified as an inhospitable environment capable of reducing numbers of various species of fungi and importantly, their ability to germinate (Edwards and Fletcher, 1988; Moody, *et al*., 1995; Parle, 1962). These findings itself do not necessarily implicate the earthworm as a vector for disease as the experiment in Chapter 4 provided no evidence to support the hypothesis that the faecal matter is infective. These findings were however important in showing that earthworms were not responsible for inhibiting spore survival or indeed viability post

ingestion. The hypothesis tested in Chapter 4 assumed that the worms faecal matter, containing viable propagules of *M. nivale* would lead to manifestations of Fusarium patch in fine turf. Limited data was obtained from this experiment due to a number of constraints in the experimental design regarding the environmental conditions present. This experiment had however relied on the earthworm proffering cast material as a result of ingesting sterilised soil inoculated with *M. nivale*. This was deemed the best method to enable enumeration of propagules post ingestion due to limited competition from fungi, normally abundant in un-sterilised soil. This experimental design has limitations as studies have suggested that earthworm casts contain high levels of microbial activity which in turn can suppress outbreaks of disease; this mechanism of pathogen suppression is based on microbial competition and hyper-paratism, and has been termed 'general suppression' (Chen, *et al.,* 1987 cited in Edwards, 2004). In field where the ingestion of *M. nivale* by earthworms may be likely, no assumptions can be made regarding the infectivity of casts based on the findings of Chapter 4. We can assume however, that these casts may contain viable propagules of *M. nivale* (Chapter 3) and that the binding agent secreted by earthworms to protect casts from desiccation has an incubation effect on organisms present within the cast. This overall process provides various fungi the propensity to survive, potentially re-establishing in a new environment. The findings of this experiment (Chapter 4) were inconclusive and would require further work; the critical observation was that spiked casts were no more infectious than spore inocula produced from the original culture of *M. nivale*. Ideally this experiment would incorporate the apparent limitations mentioned here and provide a more robust methodology that could be replicated *in natura*.

The investigation of Chapter 3 commented on earthworms as a means to decrease potential pathogenic fungi found in soils through ingestion and ejection, therefore reducing the pathogenicity respectively. The findings of Chapter 5 have however provided conclusive evidence that even if propagule presence was decreased (The data in Chapter 3 would support this) earthworm interaction increased and accelerated the rate and amount of disease manifestation (Fusarium patch) in amenity sports turf. Although these findings have concentrated specifically on *M. nivale*, it can be postulated that selective fungal consumption and dispersal could have significant implications for disease progression, clearly demonstrated by the significant differences in the manifestation of disease in the presence and absence of earthworms.

Studies have established an interaction between *Lumbricus terrestris* and saprophytic fungal pathogens especially *Fusarium* spp. Due to the feeding habits of *anecic* earthworms, fresh litter pulled into the burrows from the surface directly reduces the food source required by saprophytic fungi, leading to a reduction in numbers present (Moody, *et al*., 1995). The findings in Chapter 5 are in contrast to these views. Surface litter found in the experiment, a result of maintaining the sward to a height consistent with sports turf could well have been consumed and moved within the microcosm as a result of the habitual feeding traits of anecic earthworms. However, these actions appeared to have no consequence on reduced incidence and severity of disease, in fact quite the opposite. Although surface material including propagules may have been consumed, resulting in a lower concentration of CFUs (Chapter 3), the data implies that earthworm movement had a pronounced effect on the dispersal of *M. nivale*, as evident in the schematic representation

(Figure 5.6) and image analysis (Figure 5.7). Doube (1994) was of the opinion that manipulating the soil matrix to include earthworms that could positively affect microorganism populations, in turn reducing the severity of plant diseases and increasing plant productivity is certainly feasible. However, data presented in both Chapters 3 and 5 would ensure that there was careful consideration before elaborating on such management strategies, especially with regards to areas of fine turf. What must be considered is that the experimental microcosm was small in terms of the usual habitat occupied by anecic earthworms and required the physical inclusion of earthworms within the experimental design. Anecic earthworms are capable of travelling considerable distances with some burrows as deep as 150-240 cm with lateral movement per day much the same (Mather and Christensen, 1988 cited in Moody, *et al.,* 1995; Edwards and Lofty, 1977). Further investigation could examine how far anecic earthworms could transport infective propagules. Data obtained from a larger microcosm experiment could be used to better elucidate the complex relationships between burrowing traits and surface movement and the incidence of disease. The research could be scaled up accordingly to incorporate studies on golf courses.

6.2 The application of findings on the management of earthworms in fine turf

On fine turf e.g. golf greens and tees, earthworm castings are generally removed by means of brushing or switching, a mode of removal that relies on the breaking up of the cast and incorporation into the surface. What must be considered is these casts may contain viable propagules of *M. nivale* as ascertained in both Chapters 3 and 4. Therefore, the removal of casts by the mechanisms described is such whereby propagules can be further dispersed.

This dispersal could result in the pathogen relocating in an environment more conducive to disease manifestation. Although disease was not manifest in the experiment performed in Chapter 4, the considerations were that the host and environmental conditions were not correct. This may not be the case on amenity sports surfaces where typically outbreaks of Fusarium patch and high earthworm activity generally occur at similar times in the year, early spring and autumn. Hence, the temperature effects could well be more amenable to outbreaks of disease. If environmental conditions exist conducive to disease manifestation through earthworm castings, then the presence of earthworms in fine turf may be called into question. Completely denuding the soil matrix of earthworms is often dependent on the type of surface. On all surfaces where true ball roll is a pre-requisite e.g. golf greens, bowling greens, the removal of earthworms may be beneficial, especially considering how these surface are managed in providing the turf with all the nutrients required. However on surfaces such as football pitches or golf fairways complete eradication is neither desirable nor sensible. The removal of anecic earthworms using expellants or vermicides can have severe consequences concerning the structural stability and productivity of the soil (Bartlett, 2006). First and foremost the severity of any disease present, while only possibly linked to earthworm movement would have to be serious enough to justify denuding the matrix of earthworms. Further still, the removal of anecic earthworms could possibly result in these earthworms being replaced by other species (Bartlett, 2006). This thesis has focused primarily on the role of anecic earthworms in propagule movement; therefore, no research is available as to the effects endogeics or epigeic earthworms have on the viability and virility of *M. nivale* propagules.

The findings of this study has also provided no basis with which the augmentation of earthworms into soils conducive to disease development would be a feasible management plan for the control of disease in fine turf. It has not been possible in the scope of this thesis to understand the interaction between all earthworm species indigenous to sports surfaces e.g. golf courses and saprophytic plant pathogens present in the soil matrix. While literature provides conclusive evidence that earthworms do reduce the amounts and pathogenicity of saprophytic propagules within their surrounding environment (Toyota and Kimura, 1995), applying worms as a control measure would create larger issues whereby the consequences of high earthworm populations are more likely to outweigh the beneficial effects they induce. High density earthworm casting may become an issue affecting playability; also, this thesis has attempted to provide an insight as to the role earthworms play as vectors for disease. Until a proper understanding of the earthworm's interactions in such roles is established, it would be negligent to assume soil biota played no role in the dissemination of fungal pathogens based on the findings of this thesis.

6.3 Concluding remarks

This thesis has certainly advanced the understanding of the relationships between earthworms and fungal pathogen dispersal. It has attempted to clarify the role earthworms play in saprophytic pathogen dispersal, and although limited to specific earthworms and pathogens, has provided a basic understanding to which further extensive work can build on. The progression of this thesis has evaluated the role *L. terrestris* has as a vector for disease from start to finish; beginning with pathogen viability post ingestion, the pathogenicity of spiked cast material and ending with the effects of earthworms on

pathogen dispersal. When reporting back to the introduction of this thesis it was hypothesised that soil biota could be an integral part of the disease triangle, identified as a causal agent in disease manifestation. This thesis has provided quantitative evidence that earthworms do indeed disseminate saprophytic fungal pathogens detrimental to plant growth.

The following statements can be made regarding the impact anecic earthworms have on the dissemination of *Microdochium nivale*:

i) Based on the data obtained from Chapters 3 & 4, spores of *Microdochium nivale* do not lose viability after passage through the digestion system of *Lumbricus terrestris*. This was proven through re-isolation of propagules in the faecal matter using a series dilution technique. This therefore indicates the potential of earthworms to disperse infective propagules

ii) The earthworms digestive system does not affect the pathogenicity of propagules (Chapters 3 & 4) contained in the worms faecal matter, earthworm casts were no more potent than pure culture. This was exhibited by lack of infection in pot experiments inoculated with both spiked cast material and spore solutions.

iii) The dissemination of *Microdochium nivale* propagules is greater in the presence of anecic earthworms than if earthworms were absent in a pot microcosm containing *Agrostis stolonifera* (Chapter 5). This indicates that two modes of taxis were involved, consumption and ejection and external carriage.

iv) The rate of development of Fusarium patch is greater in the presence of anecic earthworms than if earthworms were absent in a pot microcosm containing

Agrostis stolonifera (Chapter 5). This simulation also implies that two forms of taxis were involved.

v) *Lumbricus terrestris* has no inhibitory effects on the manifestation of Fusarium patch in *Agrostis stolonifera*. This was identified in Chapter 5 whereby image analysis and leaf sampling provided conclusive evidence reporting to the dissemination of *M. nivale* propagules. Although propagule numbers may have been reduced, this was not ascertained (Chapter 5).

The finding of this thesis in relation to the existing knowledge in the field has provided a contribution in understanding the interactions between *Lumbricus terrestris* and *Microdochium nivale*. Based on the interpretations represented in this thesis there are certainly issues relating to soil fauna and the movement of pathogenic soil microbes. However, extensive further research is required to fully understand whether these interactions are indeed detrimental to sports surfaces.

Until fungicide and pesticide legislation is prohibited on amenity sports complexes, any influence that earthworms may have on disease progression and manifestation can be easily controlled. However, where the advantages of earthworms are not necessary in intensively managed turf, e.g. golf greens where all microbial activity and nutrient availability is carefully controlled, the recommendation would be to mitigate the populations of earthworms in such areas. Not only would this eliminate the potential problems associated with earthworm castings affecting playability, but also, in light of the findings represented

in this study, would negate the effects that *L. terrestris* has on the dissemination of *M. nivale* in amenity sports turf.

6.4 Further work

The following research is considered as most appropriate to provide a comprehensive study on the role earthworms play in the dissemination of *Microdochium nivale*.

i) The viability of *M. nivale* spores post ingestion was only tested on *L. terrestris* in this thesis. Moody, *et al*. (1995) reported that in testing the viability of fungal spores post ingestion through both *L. terrestris* and *A. Longa*, viability was significantly different depending on worm. Therefore further work is necessary to better elucidate the effects of different worm species indigenous to sports surface, specifically golf courses, on the viability of *M . nivale*.

ii) The data obtained in both Chapters 3 and 4 imply that the earthworms faecal matter contains viable propagules of *M. nivale*. Further research is required to understand the infectivity of such casts. An improved experimental methodology needs to be determined which better clarifies host susceptibility and which environmental conditions are required to initiate infection (Chapter 4). Research concerning the mechanistic removal of spiked worm casts on sports surfaces may be further useful in identifying optimal methods for cast removal whilst inhibiting further transit of infectious propagules.

iii) The experiment conducted in Chapter 5 showed the effects the presence of earthworms had on the incidence and severity of disease in amenity turf. The

experiment did not however, provide evidence as to the methods of pathogen dispersal. A mechanism is required that can differentiate between the methods of dissemination, whether that be through consumption and ejection or by external contamination. An experimental design could consist of enumerating CFUs collected from rinsing the earthworm intermittently during a turf microcosm experiment.

iv) A scaled up model of the experiment in Chapter 5 performed *in natura* would better clarify the distances anecic earthworms could travel whilst disseminating fungal pathogens. It is well documented that anecic earthworms have the greatest potential for pathogen dispersal. Further research is required as to the extent anecic earthworms could travel in their role as vectors.

v) This thesis has concentrated on *M. nivale*. The authors experience has however, reported unusually high earthworm activity coinciding with other disease manifestations commonplace on amenity sports turf. Therefore, further investigation is required to evaluate the effects earthworm interaction has on other fungal pathogens, most notably, *Schlerotinia homoecarpa* (Dollar spot) and *Laetisaria fuciformis* (Red thread).

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